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Presentations given at the Conference on Abnormal Functional Development of the Heart, Lungs, and Kidneys are documented in this publication. The meeting was sopnsored by the U.S. Environmental Protection Agency and was held in Asheville, NC, May 11-13, 1983.

In an attempt to carry over the flavor of informality and exchange of ideas of the conference, in this document we have grouped the articles by main subject area (General, Lungs, Heart, and Kidneys) and have concluded each grouping with an edited summary of the often lively discussion of those presentations that transpired at the conference.

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ABNORMAL FUNCTIONAL DEVELOPMENT OF THE HEART, LUNGS, AND KIDNEYS

Approaches to Functional Teratology

Edited by

Casimer T. Grabowski and Robert J. Kavlock

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TABLE OF CONTENTS

Page

List of Participants.................................... 1

Foreword (Robert J. Kavlock)............................ 5

Introduction (Neil Chernoff)............................ 7

GENERAL

Hazardous Wastes and Public Health: General Considerations and Analysis of Existing Data Sources in Florida (Robert S. Levine *et al.*).......... 13

Experimental Intrauterine Fetal Growth Retardation in the Rat: Effect of a Single Dose of Hydroxyurea or Cycloheximide on the Fetus at Term (Antonio J. Amortegui *et al.*)........................ 23

Influence of Maternal Disease on the Health and Development of the Fetus and Newborn (Euan G. Robertson)................................. 37

Assessment of the Utility of Postnatal Testing Following Prenatal Exposure to Forty Chemicals (Leon Earl Gray, Jr. *et al.*)......................... 49

Discussion Summaries--General.......................... 73

LUNGS

Normal and Abnormal Structural Development of the Human Lung (Claire Langston).......................... 83

The Regulation of Fetal Lung Development (Ian Gross)... 100

TABLE OF CONTENTS, Continued

Page

Hormones and Receptors in Developing Lung (Philip L. Ballard) 110

Effects of Prenatal Nitrofen Exposure on Postnatal Lung Function in the Rat (James A. Raub *et al.*) 125

Paracrine Regulation of Lung Growth and Maturation: The Substrate of Normal Functional Development (Barry T. Smith *et al.*) 141

Effects of Maternal Cadmium Exposure in the Rat on Prenatal Maturation of the Pulmonary Surfactant System (George P. Daston) 148

Discussion Summaries--Lungs 162

HEART

Cardiovascular Problems of the Newborn and Their Etiologies (Herbert S. Harned, Jr.) 170

The Electrocardiogram of Fetal and Newborn Rats and Dysrhythmias Induced by Toxic Exposure (Casimer T. Grabowski) 187

Electrocardiographic Studies of Developing Rodents Exposed *In Utero* to Trypan Blue (William P. Watkinson *et al.*) 209

Cardiotoxicity of Perinatal Lead Exposure (Betty J. Williams and Mary E. Abreu) 225

The Cardiac-Sympathetic Axis as a Teratological Model (Theodore A. Slotkin) 238

Discussion Summaries--Heart 250

TABLE OF CONTENTS, Continued

Page

KIDNEYS

Normal Organogenesis of the Human Kidney (Wallace W. McCrory) 257

Chemically Induced Polycystic Kidney Disease (John F.S. Crocker *et al*.) 279

The Contribution of the Kidney to the Retention of Sodium During Growth (Adrian Spitzer) 295

The Effect of Prenatal Protein-Calorie Malnutrition on Kidney Development in the Rat (Frances J. Zeman) 307

Detection of Renal Dysfunction in Neonatal Rats: Methodologies and Applications (Robert J. Kavlock and George P. Daston) 337

Neonatal Methyl Mercury Exposure: Biochemical Mechanisms in the Developing Kidney (Jorge V. Bartolome) 355

Discussion Summaries--Kidneys 363

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FOREWORD

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The U.S. Environmental Protection Agency, sponsor of the conference documented in this volume, is charged under several statutes with evaluating the relative risks and benefits involved in the use of numerous chemicals and to determine whether there is sufficient evidence to warrant restriction of their use. In the area of birth defects, risks are generally evaluated by exposing pregnant laboratory species to various doses of a chemical and examining the morphology of late-gestation fetuses. It has become increasingly apparent to researchers and regulators alike, however, that it can be highly misleading to rely solely on morphological criteria for evaluating chemically induced risks to the conceptus. Behavioral deficits in children from Minamata Bay who were exposed in utero to methyl mercury and reproductive impairments noted in females exposed to diethylstilbestrol (DES) during gestation are but two tragic examples of this possibility. It is likely that similar disturbances can be induced in other organ systems as well, but the magnitudes of such problems are generally unknown and the methods of detection in animal models not well developed. Disturbances in functional development may be accompanied by morphological lesions, or they may be much more insidious, occurring in the total absence of gross pathology. In any event, they serve to compromise the quality of life of the affected individuals. This compromise may not necessarily be evident in the newborn, but may manifest itself only much later in life, as was the case with DES.

All these items make the issue of abnormal functional development a difficult one to approach systematically. Therefore, when we laid the groundwork for the Conference on Abnormal Functional Development of the Heart, Lungs, and Kidneys, we defined three basic issues that we wanted to cover: 1) What physiological problems do human neonates generally encounter? 2) Is it possible to link these problems to any particular prenatal insult? 3) How can we best assess disturbances in functional development in animal models? To achieve these goals, we felt it was necessary to bring together researchers from three disciplines that seldom communicate with each other: clinicians, developmental biologists, and toxicologists. At first, I was a little apprehensive that we would be able to do this, but as soon as I began making phone calls I realized that there was genuine interest in a meeting of this type. Virtually everyone we sought out accepted our invitation, often in the face of difficult scheduling problems, and I am extremely pleased that we were able to attract such a distinguished group of researchers. I was somewhat amused to hear several prospective participants say that they expected to "get more out of it than would be putting into it," because that's exactly how I felt! In retrospect, the meeting does appear to have been an unqualified success in the sense that each group gained insight into the problems of the others, and we all returned to our own research environments with some new perspectives.

The accent of the conference was on informality and exchange of ideas. In an attempt to retain some of that flavor, in this volume we have grouped the articles by main subject area (General, Lungs, Heart, and Kidneys) and have concluded each grouping with an edited summary of the often lively discussion of those presentations that transpired at the conference.

INTRODUCTION

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This volume documents presentations at the Conference on Abnormal Functional Development of the Heart, Lungs, and Kidneys, a meeting sponsored by the U.S. Environmental Protection Agency (EPA) in Asheville, NC, May 11-13, 1983. A number of the scientists who attended the conference are members of EPA's Developmental Biology Division. The research mission of our Division centers upon investigations into "age-related biological phenomena." Our teratology research, which was and is responsive to the general regulatory needs of our Agency, increasingly had focused upon what is termed "functional teratology" as a means of answering questions not solvable by the use of standard test methodologies. These questions include a number raised by our regulatory colleagues concerning the significance of a wide range of effects that typically are placed in the catch-all category of "fetal" or "embryo-toxic" effects (enlarged renal pelvis, supernumerary ribs, reduced fetal weight, enlarged cerebral ventricles, hypoplastic organs, edema, etc.), or concerning that all-important value -- the "no observed effect level" (NOEL) -- that may be critical in any regulatory decision concerning an environmental agent. It became obvious that the interpretation of some development alterations induced in utero was dependent upon the ability of the affected animal to function postnatally. Furthermore, a specific organ, while not appearing to be morphologically affected, might still exhibit abnormal physiological or biochemical development in utero and/or decreased functional capabilities postnatally. As our interest in the developmental maturity of organ systems grew, we realized that a different set of end points and methodologies was needed. These end points and methodologies (or their precursors) were, in large part, found in the work of scientists in the areas of basic biochemical, physiological, and morphological development, since their work pointed to significant markers by which we could measure developmental maturity, and in methodologies used by clinicians for assessing functional deficits in humans, since the adaptation of such methodologies to laboratory animal species confers a large degree of human relevance to the laboratory studies. It was with this background that the Conference on Abnormal Functional Development of the Heart, Lungs, and Kidneys was conceived and organized by Drs. Kavlock and Grabowski. The aim was to bring regulatory teratologists, clinicians, and basic developmental biologists together to discuss areas relating to the functional teratology of a number of critical organ systems.

There is, of course, no way to adequately review the body of work that was presented at the conference and that is summarized in this volume. One can only repeat some of the highlights personally noted -- in my case, noted by a scientist who has spent virtually his entire professional life doing research in a regulatory agency.

The potential health problems that may result from our exposure to an ever-increasing array of environmental contaminants do not have to be reiterated here. Regulatory agencies are attempting, and will continue to attempt, to prevent adverse health effects due to exogenous agents by all means available to them. Any doubts about the continued reliance upon data generated in the laboratory to afford a large part of this protection should be dispelled by Dr. Levine's paper, which details the enormous difficulties inherent in the design and implementation of epidemiology studies that seek to correlate health effects (including developmental phenomena) with exposure to environmental agents. While the data obtained from studies of this type may yield important information on the extent of a general problem, they most often do not allow conclusions concerning specific environmental contaminants.

Given the continued reliance upon animal data, therefore, our primary goal in the regulatory agencies is to develop test protocols that are relevant to the human population and to develop means to accurately interpret the data generated by these protocols. To do that, I believe, we have to develop bioassays that rely upon more sophisticated end points than the production of gross terata — we have look at the development and subsequent functioning of target organs. To accomplish this goal in a rational manner, we need a thorough understanding of the comparative organ development in man and laboratory animals. Studies on normal organ development such as those presented by Dr. McCrory for the kidney are an indispensable step. Such calendars of development may be used (with appropriate care) to establish the times of chemical insult and the relevance of experimental animal and human environmental exposure periods. Another part of the basic information used is the elucidation of normal biochemical and physiological development during the embryonic/fetal and neonatal periods, as exemplified by the studies of Dr. Spitzer on the development and maintenance of sodium balance in a variety of species and by Dr. Slotkin on the biochemical and physiological markers involved in the development of neural control of the cardiovascular system. Repeatedly we are reminded of the numerous critical points, during development, that might prove sensitive to exogenous insult. Such potential critical points are quite apparent in the papers by Drs. Smith, Ballard, and Gross, whose studies highlight the complexity of growth regulation and development in the lung.

The knowledge gained in such studies, coupled with a familiarity with clinical tests, allows for design of appropriate bioassays. Dr. Harned mentions the utilization of established tests developed by neonatologists and obstetricians. One can probably also state that neonatologists have used tests developed by other "-ologists," and certainly experimental teratologists have sought, with much justification, to use tests that are known to reflect human organ function as a means of obtaining data that are easier to relate to our species. The studies reported by Dr. Daston (on the potential of cadmium to induce pulmonary hypoplasia and concomitant differences in lecithin concentration) and Dr. Raub (on functional maturity of the lung in rats exposed to Nitrofen) are examples of such studies assessing pulmonary function. Drs. Grabowski (with mirex) and Watkinson (with trypan blue) utilized ECG techniques to identify functional deficits in the hearts of both fetal and neonatal rats. It is important to emphasize that such effects, along with many others discussed in this volume, would have been missed by standard teratology tests. Both of these investigators found an imperfect match of gross structural and ECG anomalies, indicating that even normal-appearing animals may have serious functional deficits. Similarly, the studies of Zeman, Kavlock, and Bartolome indicate the potential of in utero insult to affect subsequent renal morphology and function. Dr. Zeman's work points up another factor of considerable environmental concern -- the potential of malnutrition to affect development -- and raises the question of combinatory effects of undernutrition and exposure to environmental contaminants. One other general point that should be made concerns the common instances where the NOEL for a particular organ system would be set by a functional alteration rather than a gross morphological change.

It is interesting and most significant, I think, that many of the tests used to evaluate organ function involve stressing the animal. The reserve capacity of the organism -- certainly in terms of pulmonary and renal function -- is such that often a stress is needed to make a deficit apparent. There are numerous examples of this phenomenon in the work of Drs. Kavlock, Slotkin, Raub, and others, and it is evident that simple resting-state organ function measurements are not always sufficient to indicate the presence of an organ with decreased functional capacity. I would also, however, point out that relatively simple protocols that examine postnatal growth and viability can extend our ability to note in utero effects that would be missed in standard teratology tests. Dr. Gray's work illustrates how generally simple morphological observations on the neonate can identify some effects (e.g., ablation of the Harderian gland by Nitrofen, tooth defects induced by cytosine arabinoside) that would otherwise go unnoted.

Another continual theme in the conference presentations -- and one of which both the Department of Labor and EPA are increasingly conscious -- is that development does not cease at birth. In 1979, Congress passed amendments to the Fair Labor Standards Act that allowed the Department of Labor to grant waivers for the employment of 10- and 11-year-old children in an agricultural setting, providing that appropriate reentry times were established for their protection from exposure to pesticides. It quickly became apparent that there were few data available that would allow such reentry times to be set. A number of the participants in this conference present data on the adverse effects that can be produced by exposure to exogenous agents after birth. Dr. Williams reports on cardiac arrhythmias induced by lead after exposure during the neonatal period. Drs. Spitzer and Kavlock show evidence of postnatal functional maturation in the kidney in a variety of species, including man. Dr. Crocker indicates, in fact, that a portion of his work was possible only because of postnatal development of the kidney: his effect could not be produced in utero. Dr. Langston indicates a similar occurrence in the lung. Dr. Slotkin presents data indicating postnatal developmental patterns in the enervation of the cardiovascular system (e.g., the efferent pathway developing during the first or second week post partum). It is abundantly clear, therefore, that the neonatal animal may be at special risk since its organ systems are still developing. Both as a sensitive population in itself, and as the distal portion of the developmental continuum, the postnatal animal should be included in studies designed to elucidate potential developmental toxicity. If, in humans, a cardiac developmental event occurs in midgestation or a renal event occurs during the third trimester, and if these events occur during the postnatal period in the rat, then it is clearly inappropriate to compare prenatal (or postnatal) development of these organ systems in these species. It may, however, be entirely appropriate to compare the postnatal event in the rat with the prenatal event in man if one has an adequate estimate of compound exposure in the human embryo/fetus with which to compare to the rat.

It is perhaps appropriate, at this juncture, to make two final points. The first is to acknowledge the absence of scientists who would have highlighted other organ systems. We have not attempted to deal with the reproductive system except in passing, although we are well aware of a wide variety of agents that are known to affect this system after exposure in utero. Dr. Gray's paper discusses Congo red's ability to deplete male sperm cells. There are, of course, numerous other compounds known to affect reproductive capability after in utero exposure; these include diethylstilbestrol, Clomid, busulfan, and cyclophosphamide. Likewise, we have not dealt with the immune system, although here, too, there is increasing evidence that the system is at risk during development (e.g., the effects of in utero exposure to

2,3,7,8-tetrachlorodibenzo dioxin on postnatal immune development). Two other areas of concern in which standard teratology bioassays appear to be inadequate to determine potential *in utero* insults are behavioral teratology, which has been amply covered elsewhere, and transplacental cell carcinogenesis. In terms of the latter, it is worthy of note that both the lung (by metabolites of the dialkylnitrosamines and the polyaromatic hydrocarbons) and kidney (by hydrazine and vinyl chloride) are specific targets of chemicals if these developing organs are exposed *in utero*.

The final point is that this conference highlights the potential of using peri- and postnatal studies to assess potential risk to the developing mammal. At this point in the development of regulatory testing schemes, we are using protocols that have remained essentially unchanged since the time immediately after the thalidomide episode of approximately 20 years ago. We are still relying upon gross morphological analyses of preserved fetuses, although we are well aware, as Drs. Robertson and Amortegui indicate, that many human birth defects are defined functionally. We are still attempting to maximize our test sensitivity by using high dose levels based upon maternal toxic effects, although we have known, since Paracelsus made the statement, that "All substances are poisonous, there is nothing that is not poisonous, only the dose determines that a substance does not poison." Our reliance upon high dose levels may be responsible, in part, for the fact that there are over 600 known animal teratogens but only approximately 25 known human teratogens. An increased reliance upon available kinetic information for dose selection coupled with advances in functional teratology may lead to experimental designs that will ensure greater sensitivity and accuracy in predicting interspecies responses. These advances, entailing study of functional markers perinatally, perhaps combined with a series of *in vitro* tests that examine cellular mechanisms essential for normal development and/or multispecies cultures (e.g., rat embryos and human cells or serum), may eventually form a cohesive group of improved tests upon which regulatory decisions may be based.

HAZARDOUS WASTES AND PUBLIC HEALTH: GENERAL CONSIDERATIONS AND ANALYSIS OF EXISTING DATA SOURCES IN FLORIDA

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INTRODUCTION

Florida groundwater resources are made particularly vulnerable to contamination by uncontrolled hazardous wastes because of the rock materials that serve as recharge zones to aquifers. Much of Florida is covered by highly permeable sands and soils overlying limestones. Contamination from disposal sites and other sources quickly penetrates these unsaturated sands and enters the underground reservoirs beneath. Removal of these substances is very difficult. However, an estimated 580,000 metric tons of potentially hazardous waste are generated each year within the state (Miller 1980). Groundwater, moreover, is the source of drinking water for 91% of Florida's people. Because of the magnitude of potential exposure, we have recently reviewed existing sources of environmental and public health data for Florida in order to assess the types of information available for community diagnosis of potential public health problems related to hazardous wastes.

METHODS AND RATIONALE

Survey of Existing Data

Existing sources of public health data were reviewed to identify usable denominators and numerators. Concerning the former, 1980 U.S. Census estimates were selected, with counties being chosen as the administrative unit. For the latter, fetal deaths per 1,000 births, live births of <2,500 g per 1,000 live births, and secondary sex ratio were chosen.

Selection of these indicators naturally involved rejection of others. With regard to potential denominators, county units were chosen in order to achieve the smallest feasible geographic boundaries that would provide populations large enough to produce reliable rates. Health and Rehabilitative Services (HRS) Districts were rejected because they are too big. Census tracts were rejected both because of their small size and because numerator data are not readily available. Standard Metropolitan Statistical Areas were unsuitable both because denominator data exclude large portions of the state and because numerator data are not available. Finally, U.S. Postal Service zip codes were not chosen because of concerns about the exact delineation of their boundaries and limitations as to available demographic information.

Regarding potential numerators, information on birth defects was excluded because its collection is optional throughout the state; in 1981, for example, 4 of the 11 HRS Districts did not participate at all in Florida's Congenital Defects Epidemiological Surveillance Program, and participation was spotty even among participating Districts. Fetal death and low birth weight were chosen because those data are available and because regular vital statistics procedures allow for adjustment of geographic comparisons by maternal age. Because birth and death records are not yet linked in Florida, this is not possible for the various available infant mortality parameters. Cancer morbidity data have been collected in Florida since 1981, but as yet no information is available from the Florida Cancer Data System.

The files of Florida's Department of Environmental Regulation (DER) regarding groundwater contamination were also reviewed. Data were abstracted for all sites having analytic chemical evidence of pollution as of December 31, 1982. A county-by-county log of these sites was compiled, and available information about location, history, and chemical contaminants was noted. Counties were then classified as confirmed or unconfirmed, depending on whether chemical pollution had been verified. Counties with confirmation of contamination were then grouped by the types of pollutants found (pesticides, nonpesticide organics, nitrates, heavy metals, and cyanide).

DER data were also available regarding a systematic statewide survey of drinking water supplies, and these were mapped in accordance with the maximum concentration of total trihalomethanes detected in each county (1 to 49 ppb, 50 to 99 ppb, and >100 ppb).

Finally, environmental data were sought from sources other than DER (particularly, surveys of radioactive contamination of well water).

Data Analysis for Existing Health Indicators

The detailed breakdown of population by age in the U.S. Census includes 26 categories for each sex. In addition, a cruder age distribution is provided for race. The crude race breakdown (white and nonwhite) was used in these analyses to apportion the detailed maternal age/infant sex counts into maternal age/race/infant sex counts. These were then aggregated into nine age cells (0-14, 15-24, 25-34, 35-44, 45-54, 55-64, 65-74, 75-84, and 85+ yr).

In order to adjust for differences in the composition of age, race, and sex, separate counts were tabulated for the population and the number of cases in each age/race/sex cell. For each county, incidence was then computed for each cell based on the total number of cases reported and the number of person-years. The rates were recombined using the statewide distribution of population to determine weights, resulting in an adjusted rate for each county.

For each health indicator, the adjusted frequencies were compared with the appropriate statewide value. To obtain a measure of statistical significance for the county-state comparisons, the exact cumulative binomial probability of finding the total number of cases generated by the adjusted rate was computed. Probabilities of >0.975 were taken to indicate a level of county occurrence that was significantly above the statewide level, while probabilities of <0.025 indicated a county with values significantly below statewide levels. Probabilities of >0.950 and <0.050 were classed as borderline high and low, respectively. For counties showing significant or borderline differences from statewide levels, the ratio of county to state frequencies was also computed.

For secondary sex ratio, the proportion of males to females at birth was compared for whites (51.46% males expected) and for nonwhites (50.73% males expected) (Stern 1972) using a t-test for proportions.

RESULTS

Potentially hazardous waste has been identified in at least 29 Florida counties. More than 7 out of every 10 Florida residents (7,188,940 of 9,746,324) live in confirmed counties. In general, populations in confirmed counties are larger, younger, and tend to have a greater proportion of single people, Hispanics, and blacks than populations in unconfirmed counties.

Nearly one half of the residents of Florida live in counties with drinking water supplies containing total trihalomethanes at concentrations exceeding 100 ppb. In general, counties at the other extreme (1 to 49 ppb) are less densely populated and have a lower median age level. Moreover, counties in which the drinking water supplies contain >100 ppb tend to have a greater proportion of Hispanics and a greater proportion of people living in multiple family housing units. Again, the more urban character of more highly contaminated areas is apparent.

Table 1 depicts the incidence of fetal death per 1,000 births in terms of these characteristics. For each race and sex category, the incidence of fetal death follows a similar pattern relative to maternal age: peaks occur at the extremes (especially the older maternal age groups), and the lowest levels (with the single exception of nonwhite females) occur at maternal ages of between 25 and 29 yr. In general, fetal death frequencies across the state are higher for males than for females (10.8 vs. 9.8) and for nonwhites in comparison to whites (14.8 vs. 8.6). The highest overall rate is for nonwhite males (15.5), and the lowest is for white females (8.1).

Table 2 presents the occurrence of low birth weight per 1,000 live births for the same age/sex/race classification. Peaks occurred at the extremes of maternal age (i.e., 138.9 low birth weight infants per 1,000 live births among mothers of <14 yr of age, and 160.7 among mothers of >50 yr of age), with the low value (64.2) for 25- to 29-yr-old mothers. The single exception to the steady increase from the 25- to 29-yr-old level is the somewhat lower value for 45- to 49-yr-olds (93.8) relative to 40- to 44-yr-olds (103.3). Age-specific patterns within each sex-race group generally reflected those for the statewide levels. For both whites and nonwhites, however, levels among female infants were higher than those among male infants.

Table 1. Fetal Deaths per 1,000 Total Births, State of Florida, 1978-1980

	Infant Sex and Race				
Maternal Age (yr)	White Male	White Female	Non-white Male	Non-white Female	State Total
<14	31.6	24.8	25.4	18.3	23.5
15 - 19	10.6	10.4	14.1	14.2	12.1
20 - 24	8.4	7.6	14.2	11.8	9.3
25 - 29	7.8	6.1	13.6	12.2	8.2
30 - 34	8.7	9.3	20.3	20.8	11.2
35 - 39	18.5	12.0	25.0	18.2	16.9
40 - 44	31.6	19.6	14.2	37.4	25.7
45 - 49	-	85.7	105.3	62.5	58.8
50+	200.0	200.0	481.5	461.5	363.6
Total	9.1	8.1	15.5	14.2	10.3

Table 2. Live Births of <2500 g per 1,000 Live Births, State of Florida, 1978-1980

	Infant Sex and Race				
Maternal Age (yr)	White Male	White Female	Non-white Male	Non-white Female	State Total
<14	110.2	76.3	148.4	160.2	138.9
15 - 19	79.3	82.6	132.0	145.3	105.5
20 - 24	56.2	65.9	107.3	133.6	76.8
25 - 29	51.1	56.0	97.0	112.3	63.7
30 - 34	50.3	60.2	94.9	111.2	64.2
35 - 39	73.5	77.7	82.4	111.2	80.9
40 - 44	87.4	107.4	109.5	122.2	103.3
45 - 49	93.8	93.8	58.8	133.3	93.8
50+	166.7	62.5	142.9	285.7	160.7
Total	58.0	65.1	111.0	129.9	77.3

Table 3 is a summarized geographic comparison for health indicators in counties where statistically high or borderline high values were obtained for fetal death and/or low birth weight or where statistically high or low values were obtained for secondary sex ratio. Specifically, the table shows the ratio of county to state levels for fetal death rate and rate of low birth weight as well as the secondary sex ratio. While these ratios are statistically unusual, they are almost uniformly below 1.50. Only two counties show values in excess of 2.00, and these are areas with relatively small populations.

DISCUSSION

Clearly, a large amount of information about hazardous wastes has been assembled by environmental and health agencies in Florida. From the standpoint of attempting to relate environmental contamination to adverse human health effects (including functional birth defects), however, it is

Table 3. Summary of Statistically High and Borderline High Florida Counties for Health Indicators: Ratio of County to State Values for Age/Sex/Race-Adjusted Frequencies of Fetal Death and Low Birth Weight and Ratio of Live-Born Males to Females Among Nonwhites[a]

Health Service District and County	Fetal Death	Low Birth Weight	Sex Ratio
District 1			
Escambia			
Okaloosa			
Santa Rosa			
Walton	2.43		
Districts 2a and 6			
Bay			
Calhoun			
Franklin			1.37
Gadsden			
Gulf			
Holmes			
Jackson	1.38		
Jefferson			
Leon			
Liberty			
Madison	1.92		
Taylor			
Wakulla			
Washington			
District 3a			
Alachua			
Bradford			
Columbia			
Dixie			
Gilchrist	3.50	1.43	

(continued)

Table 3. (continued)

Health Service District and County	Fetal Death	Low Birth Weight	Sex Ratio
Hamilton			
Lafayette			
Levy			
Putnam			
Suwanee	1.80		
Union			
District 3b			
Citrus			
Hernando			0.37
Lake			
Marion		1.11	
Sumpter			
Districts 7a and 7b			
Brevard	1.17		
Orange			
Osceola			
Seminole			
Districts 4a and 4b			
Baker			
Clay			
Duval			
Flagler			
Nassau	1.63		
St. Johns			1.26
Volusia			
Districts 5, 6, and 8			
Collier			
Pinellas			

(continued)

Table 3. (continued)

Health Service District and County	Fetal Death	Low Birth Weight	Sex Ratio
Charlotte		1.19	
Desoto			
Glades			
Hardee			
Hendry	1.47		
Highlands			
Hillsborough		1.09	
Lee	1.27		0.48
Manatee			
Pasco	1.41	1.15	
Polk			1.11
Sarasota			
District 9			
Indian River	3.80		
Martin			
Okeechobee			
Palm Beach	1.17	1.09	
St. Lucie			1.19
District 10			
Broward		1.08	
District 11			
Dade	1.07		
Monroe			

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[a] None of the county values for whites differed significantly from the expected percentage of 51.46 for live-born males; the expected value for nonwhites was 50.73% (Stern 1972).

also clear that this information can only represent a tentative first step. The environmental data are not very useful for defining exact populations exposed to pollutants, do not indicate the long-term course of human exposure, fail to describe the actual human burdens of environmental contaminants, are not specifically related to health data, and (because of the nonsystematic nature of current groundwater sampling) provide no assurance that areas currently listed as negative are truly uncontaminated.

The health data are nonspecific (Hook 1981) and, in certain key instances, simply absent. Further, descriptive health data can be misleading on several counts. Classification of areas as not statistically different or low relative to the rest of the state, for example, does not necessarily establish that no problem exists. On the contrary, such results are consistent with an exposure that affects a relatively small number of people within a county, whose effects may be balanced or reversed by protective effects within the region, or whose effects have been acting for too short a time to become manifest. Similar concerns apply with respect to the low ratios for counties relative to the state among those areas with statistically high health indicators; these ratios, however, may serve to buy time for the careful consideration of exactly what kinds of data should be collected to provide more definitive answers. Such deliberations need to begin promptly and need to include ways of obtaining specific measurements of human exposure and human disease relative to hazardous waste sites.

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EXPERIMENTAL INTRAUTERINE FETAL GROWTH RETARDATION IN THE RAT: EFFECT OF A SINGLE DOSE OF HYDROXYUREA OR CYCLOHEXIMIDE ON THE FETUS AT TERM

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INTRODUCTION

Growth failure in young children is a major medical and public health problem of worldwide importance, especially in the developing countries (Viller, Belizan 1982). Growth failure may occur either during uterine development (Gruenwald 1963; Aust NZ J Obstet Gynecol 1969) or in postnatal life (Winick, Noble 1965; Winick, Noble 1966; Winick, Noble 1967).

The importance of intrauterine fetal growth retardation (IUGR) as a major cause of perinatal mortality and of growth failure has been appreciated only in recent years (Gruenwald 1963; Starfield et al. 1982; Van den Berg, Yerushalmy 1966). The definitions are still imprecise and, as expected, the etiologies are complex. Recognition of the increased perinatal mortality (Aust NZ J Obstet Gynecol 1969; Starfield et al. 1982) and morbidity and of the permanent physical (Chase et al. 1972; Winick 1969; Winick 1971) or mental retardation (Chase, Martin 1970; Scrimshaw 1967; Stoch, Smythe 1967; Randt, Derby 1973) that may follow intrauterine growth retardation has stimulated the study of the pattern, rate, and sequence of normal growth and development.

Three phases of growth have been described (Winick, Noble 1965). Early in embryonic development, growth occurs exclusively by cell division. Later, growth is accomplished by increase in cell size and cell number. Finally, in many tissues, increase of cell number ceases and further growth is limited to increase in size of preformed cells.

The great variety of types of IUGR depend upon the nature of the various stimuli and the time and duration of their interference with growth and development. Multiple factors are responsible for the IUGR syndrome: spontaneous genetic mutations, radiation, toxicity of drugs or chemicals, microbes, and maternal factors such as diabetes, hypertension, alcoholism, cigarette smoking, and inadequacy of nutrition, whether due to inappropriate diet or to interference with effective maternal-placental circulation or placental transport. In general, the earlier in gestation and the more prolonged the insult, the more irreversible and unretractable its results are likely to be. By the same token, IUGR due to causes later in pregnancy should be more amenable to prevention and control. Effects range from death due to faulty embryogenesis to survival with malformations or growth retardation secondary to altered cellular differentiation or growth. It has been estimated that 22 million infants were born with low birth weight in 1975, 93% of them in poor areas of the world (Viller, Belizan 1982). Growth retardation occurring early during periods of development may have permanent adverse effects on the level of physical and mental attainment.

The general theory is that any decrease in size of a cellular tissue or of a whole organism is due to deficit in cell number and/or to decrease in cell size. A deficit in cell number, resulting from an insult during a critical phase of organ growth or development *in utero* and/or in extrauterine life, may be permanent and may result in decrease in organ size and function. A decrease in the size of cells, on the other hand, if the cells remain endowed with normal nuclear genetic material, may be a potentially reversible defect.

Most experimental animal models (Evans *et al*. 1983) for producing IUGR by diet restriction (Zamenhof *et al*. 1968;

Chow, Lee 1964; Platt, Stewart 1968; Kirsch et al. 1968; Stewart, Sheppard 1971), vascular ligation (Wigglesworth 1964; Myers et al. 1971; Hill et al. 1971) or obstruction (Creasy et al. 1972), superovulation (Evans et al. 1981), extrinsic stress (Hensleigh, Johnson 1971; Kolleel et al. 1979; Levistsky et al. 1977), hypoxia (Van Geiju et al. 1980), uremia (Andrews et al. 1970; Nitzan et al. 1979), or partial placental ablation (Myers et al. 1971) have a prolonged or chronic effect during fetal development and may be expected to produce a mixed pattern of retardation due to reduction in both cell number and cell size.

The basis of the model used here rests upon specific inhibition of DNA or of protein synthesis in the fetus for a brief period by administration to the maternal rat of single doses of antimetabolic drugs.

The present experiments were undertaken to provide confirmation of a previous model (Klionsky, Wigglesworth 1970), to add observations on additional tissues, and to provide a baseline for survival experiments in which the effect of this model of IUGR can be evaluated in postnatal individuals.

MATERIALS AND METHODS

Pregnant Sprague-Dawley rats were obtained commercially (Zivic-Miller, Pittsburgh, PA). The duration of pregnancy was reckoned in days elapsed from the presumed time of fertilization at 0100 h on the day when a vaginal plug was detected.

Three groups of 10 animals were used. Animals in the first group were injected with a single 600-mg dose of hydroxyurea (approximately 1800 mg/kg body weight) in 3 ml isotonic saline at approximately 9 a.m. on the 15th day of pregnancy.

Animals in the second group were injected with a single 0.2-mg dose of cycloheximide (approximately 0.75 mg/kg body weight) in 1 ml isotonic saline at approximately 9 a.m. on the 18th day of pregnancy.

Animals in the third group, designated as controls, were injected with 3 ml or 1 ml isotonic saline at times

corresponding to the drug injection. All injections were intraperitoneal.

All animals were anesthetized and killed by cervical dislocation at approximately 9 a.m. on the 21st day of gestation. A caesarean section was performed, and three fetuses from the midline were selected in a standard manner, weighed in a torsion balance, and decapitated. If the number of fetuses in the two horns differed, two fetuses were chosen from the horn with the larger number. Brain, liver, heart, kidneys, and placenta were dissected, trimmed, and weighed. The organs were homogenized and stored at -70°C. One fetus from each litter was dissected, and representative sections were fixed in 10% Stieve's solution for histological investigation.

In a second experiment conducted in a similar fashion, one group of animals was allowed to deliver and develop. Animals were weighed after birth, allowed to grow to 28 d, and then anesthesized and sacrificed. The total body and organ weights were obtained and chemical analyses of heart, liver, brain, and kidneys performed to determine values of DNA, RNA, proteins, and glycogen.

Biochemical Methods

Protein, DNA, and RNA were analyzed with methods derived from Wannamacher et al. (1965) and Munro and Fleck (1966). All centrifugations were performed at 2000 rpm for 20 min at 4°C. Carbohydrate was tested by the phenolsulfuric acid method (Hodge, Hofreiter 1962).

Standards were prepared from bovine albumin (Ortho Diagnostic, Raritan, NJ), calf thymus DNA (Sigma Chemical, St. Louis, MO), and glycogen (Fisher Scientific, Pittsburgh, PA). Cell number was determined from the measured DNA. Calculations assumed a constant value of 6.4×10^{-12} g DNA in the nucleus of every diploid rat cell (Enesco, Leblond 1962). For other components, the quantity per organ, the concentration in fresh tissue, and the quantity per cell were calculated.

Statistical Methods

Statistical analyses were performed for each of the 14 parameters listed in the tables for each organ, including maternal and fetal body weights.

A statistical evaluation of the results was performed by nested analysis of variance (Sokol, Rohlf 1968). Tests of significance were made for the overall treatment effect and for the orthogonal pair of contrasts: 1) Control versus two drug treatments, and 2) cycloheximide verus hydroxyurea. Comparisons between individual treatment effects and the controls were calculated as F values using the pooled error variance for rats.

RESULTS

The single dose of hydroxyurea or of cycloheximide did not have a significant effect on the weight of the maternal rat. Both cycloheximide and hydroxyurea treatments resulted in a significant ($P < 0.0001$) decrease in fetal body weight at the time of caesarean delivery (Fig. 1). Although the total fetal body weights did not differ significantly in the two experimental groups, many tissues of the hydroxyurea-treated animals were significantly smaller than those from the cycloheximide-treated animals.

Organ Weights

The pattern and degree of response varied markedly from organ to organ (Fig. 1). In brain, liver, and kidney, both treatments resulted in significant decrease of organ weight as compared to the control; the weight was more significantly decreased in hydroxyurea-treated animals than in those treated with cycloheximide.

The patterns of response of heart and placenta differed in several ways from those of the other organs. The placental weight did not vary significantly between the two groups, but was lower in the mothers treated with cycloheximide. The heart weight, in contrast to the generally observed pattern, was significantly less after cycloheximide treatment than after hydroxyurea.

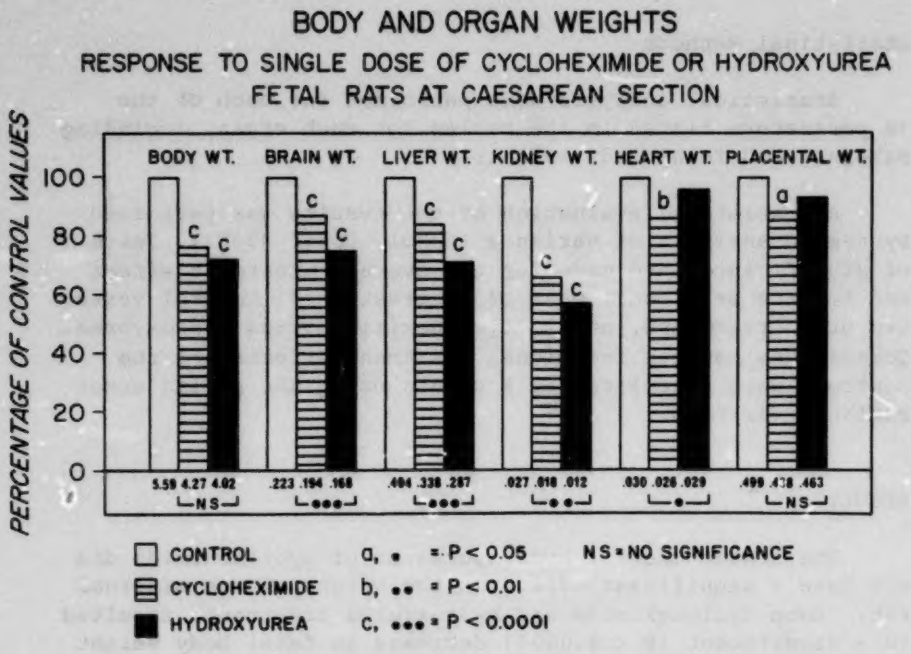

Fig. 1. Effect of a single maternal dose of cycloheximide (on Day 18) or of hydroxyurea (on Day 15) on body and organ weights of fetal rat at caesarean delivery at term.

Organ Biochemistry

The basic data for the individual organs are presented in Table 1 and illustrated in Fig. 2.

Since changes in DNA (cell number) are of prime importance, the varied pattern of responses deserves special attention. Hydroxyurea treatment of the pregnant rat resulted in inhibition of DNA synthesis and therefore in numbers of liver, kidney, and brain cells in the offspring. In the placenta, there was no significant change in DNA; there was an apparently paradoxical increase in the amount of DNA in the heart. In contrast, the fetuses subjected to cycloheximide had a significant decrease in DNA and cell number only in heart, kidney, and placenta.

Table 1. Effect of a Single Dose of Cycloheximide or Hydroxyurea During Pregnancy on Weight and Chemical Composition of Rat Organs at Caesarean Delivery[a]

		WEIGHT /ORGAN mg.	PROTEIN /ORGAN mg.	RNA /ORGAN mg.	DNA /ORGAN mg.	CHO /ORGAN mg./gm.	PROTEIN CONC. mg./gm.	RNA CONC. mg./gm.	DNA CONC. mg./gm.	CHO CONC. mg./gm.	CELL NO. $x10^6$	CELL PROTEIN $x10^{-12}$ gm.	CELL RNA $x10^{-12}$ gm.	CELL CHO $x10^{-12}$ gm.	CELL WEIGHT $x10^{-9}$ gm.
BRAIN	Control	.223	13.3	1.07	.898	3.78	59.6	4.82	4.05	17.1	145	93.3	7.56	26.7	1.58
	Cyclo	.194 ***	12.2	1.01	.868	3.25	63.2	5.19	4.49	16.8	140	88.1	7.20	22.3	1.39 *
	Hu	.168 ***	11.0 **	.840 ***	.694 ***	3.70	65.5	4.99	4.13	22.1 ###	112 ***	98.8	7.56	33.4 ##	1.53
LIVER	Control	.404	44.0	5.21	2.30	36.5	109	13.0	5.73	90.7	371	120	14.2	99.8	1.11
	Cyclo	.338 ***	38.8	4.57 *	2.08	37.8	117	13.7	6.18	112	336	119	13.9	115	1.02
	Hu	.287 ***	32.8 ***	3.94 ***	1.90 *	27.9	115	13.8	6.67 #	96.8	306 *	111	13.4	102	988
KIDNEY	Control	.0267	1.98	.239	.183	.0860	74.9	9.27	7.02	3.61	27.8	69.7	8.49	3.36	1.05
	Cyclo	.0177 ***	1.47	.177 *	.108 **	.072 *	85.5	9.98	6.38	4.10	17.5 **	111	11.2	4.66 #	1.28
	Hu	.0123 ***	1.31	.153 **	.111 **	.0583 **	108	12.5 #	9.15	4.85	17.8 **	80.5	8.94	3.41	.766
HEART	Control	.030	2.77	.216	.104	.539	94.1	7.53	3.52	18.0	16.8	[illegible]	13.3	32.3	1.87
	Cyclo	.026 **	2.27 *	.173	.073 **	.384 *	89.6	6.84	2.95	15.0	11.8 **	220	15.9	36.9	2.55 #
	Hu	.029	2.75	.219	.125 #	.523	95.4	7.60	4.34 #	18.2	20.1 #	138	11.0	26.4	1.45
PLACENTA	Control	.499	66.0	2.72	1.22	12.6	132	5.49	2.48	25.0	196	346	14.2	66.3	2.60
	Cyclo	.438 *	56.4	2.53	1.05 *	10.1	130	5.83	2.46	22.7	169 *	337	15.1	59.9	2.65
	Hu	.463	55.2 *	2.30 **	1.13	10.8	120	5.02	2.49	23.3	182	314	12.9	67.2	2.61

o = P<0.10   * or # = P<0.05   ** or ## = P<0.01   *** or ### = P<0.001

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[a] Each data point represents the average of 30 values obtained by analysis of 3 fetuses from each of 10 maternal rats. Data are expressed separately for each organ.

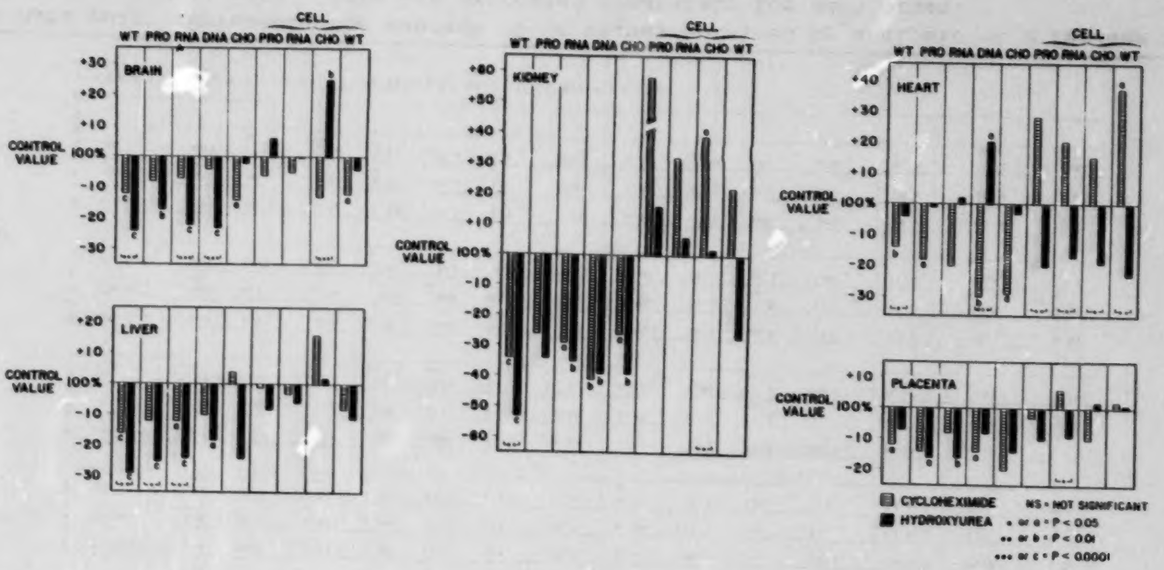

Fig. 2. Comparisons of effects of a single maternal dose of cycloheximide (on Day 18) or of hydroxyurea (on Day 15) on percentage weight and composition of rat fetal organs at caesarean delivery at term.

Because of the marked variation in organ response, there can be no attempt at general description of the biochemical results. Data for each organ will be considered separately.

Brain

The brain shows a relatively pure decrease in cell number following hydroxyurea treatment and a decrease in cell weight after cycloheximide. Following hydroxyurea, all of the primary parameters except carbohydrate are significantly decreased. Consequently, there is an apparent increase in the amount of carbohydrate per cell. Organ weight is the only primary parameter affected to a significant degree by cycloheximide.

Liver

In the liver, all the primary parameters show a decrease with hydroxyurea, but the 24% decrease in liver carbohydrate is not statistically significant. Cycloheximide produces a highly significant decrease in organ weight but only a decrease on the order of 5% in the amount of RNA per organ.

Kidney

With the kidney, the basic parameters show a trend to decrease in both the hydroxyurea and the cycloheximide groups. This trend is not statistically significant for protein. Ratios estimating cell size tend to increase.

Heart

Effects upon the heart are less marked than in the other tissues studied. There is no decrease in any of the primary parameters after hydroxyurea; indeed, there is a paradoxical increase in DNA and cell number. The reaction pattern in the heart is unusual in that all primary parameters are decreased more after cycloheximide than after hydroxyurea. Also, there is a paradoxical increase in all of the ratios that measure cell size. All of these factors are statistically significant for the comparison between cycloheximide and hydroxyurea.

Placenta

The primary parameters show a trend to decrease from control levels after both cycloheximide and hydroxyurea.

DISCUSSION

These experiments are based upon the hypotheses that a transient inhibition of DNA synthesis during critical periods of organ development would result in an irreversible deficit in organ cell number, and that a transient inhibition of protein synthesis might result in a reversible decrease in cell size (Wannamacher et al. 1965). Single doses of hydroxyurea -- an inhibitor of DNA synthesis (Munro, Fleck 1966) -- or of cycloheximide -- an inhibitor of protein synthesis -- were given to rats during gestation in quantities and at times most likely to produce a decrease in body weight to the extent of 2 to 3 standard deviations and expected deviant patterns of cell number and of cell size.

Additional unpublished experiments (Amortegui, Klionsky 1983) based upon longitudinal observations of fetuses permitted to deliver and develop indicate that, in general, the growth retardation produced by hydroxyurea is permanent whereas that of cycloheximide is reversible.

Mechanism

Profound changes in total fetal body weight and in the weights and chemical compositions of organs occurred with single doses of drugs that had transient metabolic inhibitory effects on the fetuses with no apparent clinical effect on the weight or behavior of the mothers. The animals used in the present experiments cannot be compared with those used in England (Klionsky, Wigglesworth 1970), since they were of different strains and different weights as well as maintained on different diets. Nevertheless, the data obtained for the brain and the liver in the current experiments agree with the values found in the previous studies. Incorporation experiments with ^{14}C-labeled leucine following cycloheximide indicate that maternal and fetal tissues alike have a peak of about 80% inhibition of protein synthesis, with a return to normal levels about 6 h later. Similar incorporation experiments with ^{14}C-labeled thymidine (Bhan et al. 1983) following hydroxyurea showed, as expected, no significant inhibition of DNA synthesis in maternal liver, while fetal DNA synthesis, which was reduced to about 10% of normal, returned to control levels within 12 h. These data emphasize that the developing fetus may be seriously affected by transient metabolic insults that have no deleterious effect upon the mother.

These experiments do not provide a general model for all organs for the creation of a pure cell number depletion with hydroxyurea or of a pure cell size deficiency with cycloheximide. Different organs have their own patterns and chronology of development and respond differently to the fixed time and duration of metabolic interference created by a given set of experimental conditions.

A timed series of more detailed chemical and ultrastructural experiments would be be necessary to evaluate the sequence of dynamic events occurring in the response to injury. In the present studies, the chemical data were obtained from fetal samples taken, respectively, 3 or 6 d after brief periods of metabolic protein or DNA synthetic blockade. These measured responses may reflect more than the direct response to injury and may be complicated by the effects of mechanisms of compensation and recovery.

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INFLUENCE OF MATERNAL DISEASE ON THE HEALTH AND DEVELOPMENT OF THE FETUS AND NEWBORN

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INTRODUCTION

Perinatal mortality rates have fallen by nearly 50% in the past 25 years. About half of those deaths occur before the birth of the infant, and the remainder are neonatal deaths. The improvement in perinatal mortality and the accompanying reduction in family size which is presently occurring in this country have led to the anticipation that the majority of infants who survive the birth process will also remain alive and healthy after the neonatal period. The major cause of mortality at the present time is prematurity. Once the risk of the premature infants has been removed, there remains a small group of fetuses and neonates who will die when they approach maturity. Some of these infants have severe structural abnormalities; others, however, have functional defects which can be related to the lifestyle or to the health of the mother. These functional defects may be incompatible with life or may produce effects which lead to severe functional deficits in later life.

In prenatal care the present major thrust must be toward reducing the perinatal mortality rate due to prematurity. As our efforts become more successful, they begin to focus on those infants who reach term and then die as the result of some health-related problem to which the mother has been exposed during the pregnancy. In the majority of instances the cause is well understood -- for example, in the child of the diabetic mother or in the baby whose mother has antibodies to the rhesus factor. However, in cases where the baby has a functional deficit in heart, lung, or kidney function for which the cause is not known, the possibility of environmental toxin exposure must be raised. Although the effects of some toxins are recognized (for example, tobacco and alcohol), the effects of environmental pollutants are in the main not understood or recognized. This review will concentrate on the effects of maternal exposure to certain diseases and the resulting effect on functional development in the fetus, and it will speculate about effects which may be responsible for some unexplained cases of deaths and other severe disease states in the infants at birth.

FACTORS AFFECTING FETAL GROWTH *IN UTERO*

The effects of environmental and other factors on the fetus and embryo depend upon the time in gestation at which exposure occurs. If the embryo is exposed at an undifferentiated stage, the outcome is embryonic death and abortion. During the stage of organogenesis, structural effects can occur which may be life threatening and may simply disrupt the social function of the individual. After organ development is complete, exposure to an adverse environment will lead to retardation of growth in the fetus and to the delivery of a baby which is small for gestational age. Although exposure to viral and chemical teratogens will produce these effects, most cases of intrauterine growth retardation which are seen in clinical medicine result from health-related problems preexisting in the pregnant mother. To detect growth retardation, the normal pattern of fetal growth *in utero* must be known. Several nomograms are available which enable the predicted weight of the fetus to be compared with an ideal weight for that gestational age. A disadvantage of these nomograms is that the influence of geographic factors on birth weight was not considered when the nomograms were developed. The best example of this is the effect of high altitude, which causes chronic hypoxia and slowing of growth. One of the original nomograms, which is widely used for detecting growth retardation, was developed in Denver at 5,000 ft. above sea level (Lubchenko *et al*. 1963). Data in this nomogram are not appropriate for comparison with infants born at sea level. Ideally, each health center making comparisons should either develop its own normal growth tables, or utilize those based on data from areas with a similar climate and environment.

The main influences on fetal growth can be categorized:

1) Constitutional: A small mother who belongs to a race of individuals who are generally of small stature is liable to give birth to an infant which is small for gestational age by comparison with a group of larger stature.
2) Vascular Disease in the Mother: This is particularly related to those patients who suffer from conditions such as hypertension -- both chronic and pregnancy induced, chronic renal disease, and heart disease and arteriopathy as the result of diabetes.
3) Exposure to Toxic Substances: Tobacco smoking and the chronic consumption of alcohol have appreciable effects on the growth of the infant, and chronic exposure to other unknown environmental teratogens may produce appreciable effects on fetal growth.
4) Exposure to Viral and Other Chronic Infections During Pregnancy: Fetal infections with cytomegalovirus, rubella, and possibly other viral agents beyond the stage of organogenesis are responsible for intrauterine growth retardation. Exposure to toxoplasmosis can also have a similar effect.
5) Exposure to Environmental Pollutants: Although the majority of infants with intrauterine growth retardation have some identifiable factor present in the maternal history, there is a group of significant number in which the infant is growth retarded and yet the mother's health appears normal. This group, in which exposure to environmental pollutants may play a profound and yet unidentified role, is worthy of further investigation.

The identification of fetal growth retardation rests mainly upon detection of the risk factors which are known to be associated with retarded fetal growth. Subsequently, careful evaluation of fetal growth in utero is necessary. Simple measurement of the uterine fundal height will indicate whether the uterus is growing within normal limits. If there is a discrepancy is size from the expected size for age, then sonographic measurements of the fetus are essential to determine whether growth is occurring normally. The fetus may demonstrate different patterns of growth retardation. There are two main forms of growth retardation, the symmetric, where there is generalized retardation of growth, and asymmetric, in which the head growth is normal but the trunk grows slowly. Because either type of growth retardation may occur, it is necessary to do multiple measurements on the fetus and not rely solely on biparietal diameter measurements. Symmetric growth retardation usually occurs as the result of exposure to external agents such as viruses and tobacco and alcohol. Asymmetric growth retardation is found in patients with severe vascular disease, particularly in those patients with chronic hypertension which has been uncontrolled during the pregnancy and has resulted from chronic renal disease in the mother. The effect of vascular disease is to reduce blood flow to the choriodecidual space and thus to deprive the placenta of both oxygen and essential nutrients. The infant thus becomes "starved" *in utero* and tends to be especially liable to hypoxic insults during the later stages of pregnancy and labor. The severely growth-retarded newborn is unusually susceptible to hypothermia because of the inability to store fat during the course of pregnancy. These infants may also develop metabolic derangements, particularly hypoglycemia. They are also liable to demonstrate bleeding diatheses as the result of the liver's inability to produce clotting factors. A main risk to these infants, however, is that growth retardation is unrecognized, and placental failure occurs so that the infant is stillborn.

The subsequent effects of intrauterine growth retardation depend very much upon the cause. If growth retardation is due to a maternal factor such as hypertensive disease and poor vascularity and the infant has asymmetric growth retardation, the fetus is very likely to grow normally and demonstrate few neurologic and other abnormalities during subsequent development (Lubchenko 1976). On the other hand, if the infant has suffered symmetric growth retardation as the result of the exposure to a teratogen or toxin, there is considerable evidence that these infants are at high risk for developing subsequent neurologic abnormalities and are liable to grow only slowly after birth (Fancourt et al. 1976). The effects of growth retardation are seen in all systems of the body and represent the extreme effects of exposure to a bad environment on a developing fetus. The keys to reducing growth-retardation effects are early detection and early intervention to remove the fetus from the adverse environment as soon as possible. Although many disease states will cause fetal growth retardation, several of these will also cause specific organ defects which are obvious only after birth, even in the well controlled, well treated individual.

The Effects of Maternal Diabetes on Fetal Growth and Development

Uncontrolled maternal diabetes produces an effect opposite to fetal growth retardation in that the fetus grows excessively and is macrosomic. Diabetes in the mother also produces functional effects, particularly in the lungs, which affect the ability of the newborn infant to survive. The classic description of the infant born to a diabetic mother is that of a plump, plethoric, cushingoid infant with an appearance of obesity and overhydration. This initial impression is misleading, since in fact there is hypertrophy and hyperplasia in most organs, with the exception of the brain, and reduction in tissue water throughout the body. The mechanism responsible for macrosomia of the fetus is not clear. One hypothesis is hyperglycemia in the fetus, which causes stimulation of the fetal pancreas to produce excessive insulin, which in turn enhances fetal glycogen synthesis, lipogenesis, and protein synthesis (Hill 1978). Although it is difficult to get direct evidence of fetal hyperinsulinemia, there is no doubt that these infants are born with hypertrophy and hyperplasia of the pancreatic islets and that an increase in the number of beta cells is occurring in these infants. Another factor frequently found in uncontrolled diabetes is polyhydramnios -- the excessive production of amniotic fluid. This in association with the macrosomia in the fetus can produce extreme enlargement of the maternal abdomen during the course of pregnancy. A mechanism for the excessive fluid production is unknown, but it is assumed to be related to the effect of the high circulating-blood-sugar levels upon the fetus, which in some way causes disruption of the normal circulation of amniotic fluid within the fetoplacental circulation.

These macrosomic infants have a poor capacity for survival because they are metabolically unstable at birth, suffering from very rapid shifts in blood sugar. A specific effect which is seen even in the absence of macrosomia in the diabetic infant is the effect on lung maturation. There is an increased incidence of hyaline membrane disease (HMD) and respiratory distress syndrome (RDS) in the neonates born to diabetic mothers, even when the diabetes has been well controlled (Frantz, Epstein 1978). HMD in the neonate results from the delivery of the fetus at a time when there is insufficient synthesis, storage, or release of surface-active substances (grouped as pulmonary surfactant) in the alveolar type II cell of the lung. These substances reduce surface tension at the air/alveoli interphase and are necessary for normal breathing and gas exchange. When surfactant is absent or deficient, atelectasis results and HMD appears. Normally the surface-active agent can be detected in the amniotic fluid in the last 4 weeks of pregnancy. The commonest clinical test used to detect these phospholipids is the lecithin/sphyngomyelin ratio. This test, however, has been shown to be ineffective in predicting pulmonary surfactant maturity in the diabetic, which has led to a more detailed investigation of the nature of phospholipids in the amniotic fluid of the pregnant diabetic. Desaturated phosphatidylcholine accounts for 45% of the surface-active material isolated, but recent data suggest that phosphatidylglycerol and phosphatidylinositol may play an important role in lowering surface tension and may be deficient in infants with HMD (Hallman, Gluck 1976). Synthesis of these compounds appears to be delayed in the infant of the diabetic mother, but the mechanism for this delay is not known. It is possible that, since cortisol appears to activate the enzymes of surfactant synthesis, insulin may in some way antagonize this action (Smith et al. 1975).

Many of the adverse effects of diabetes can undoubtedly be controlled by careful manipulation of blood sugar and insulin levels during the course of pregnancy. Frequent blood sugar monitoring and alteration of insulin dosage can produce blood sugar levels which mimic those in the normal subject, and this appears to reduce considerably the risk of macrosomia. However, the adverse effects of diabetes or possibly the administration of insulin on the ability to synthesize phospholipids within the lung have not been reduced to the same extent, and RDS and HMD remain serious causes of morbidity in these infants. However, RDS also occurs in babies who are born at term to mothers who are not diabetic. Although this RDS type occurs infrequently, it can be severe and life threatening. It is not known what agents reduce the ability of the lungs to expand at birth in this infant group. It is possible that exposure to environmental pollutants or mutagens may have played some role in the abnormal functional development.

Hydrops Fetalis

Another group of infants at risk from death or morbidity as the result of a maternal process are those babies born to mothers who have conditions which predispose to destruction of red cells in the fetal circulation. The classic example of this is rhesus isoimmunization, but there are other causes, particularly related to antibodies to other blood-group factors, and to babies born to mothers with thalassemia. A small but recognizable group exists in which hydrops fetalis occurs without any obvious immune factor to explain the occurrence of the disease.

The original description of hydrops fetalis was made as the result of an understanding of the development of maternal antibodies to the rhesus factor. If a mother becomes sensitized to the rhesus factor and produces antibodies, these readily gain access to the fetal circulation. In rhesus-positive infants such antibodies are absorbed onto the Rh-positive red cells and in addition exist in a free form in the infant's serum. The absorbed antibodies act as hemolysins, leading to destruction of the fetal red cells. The effect is progressive anemia in the fetus, but there are also widespread effects on the fetal circulation and other organ systems which are not readily explicable by the degree of fetal anemia alone. The pathologic changes in the organs of the fetus and the newborn vary with the severity of the process. The severely affected infant may show considerable subcutaneous edema as well as effusions into the serous cavities. In these cases the placenta is also markedly edematous and is appreciably enlarged with prominent cotyledons and edematous villi. Excessive and prolonged hemolysis stimulates marked cellular hyperplasia of the bone marrow as well as large areas of extramedullary hematopoiesis, particularly in the spleen and the liver. Fatty degeneration of the liver parenchyma occurs as well as deposition of hemosiderin and engorgement of the hepatic canaliculi with bile. There may be cardiac enlargement and pulmonary hemorrhage. Heart failure, however, at least at the outset, does not appear to play a prominent role in the development of ascites. There appears to be some other immune effect on the vascular wall in the fetus, and these infants develop severe hypoalbuminemia, which leads to excessive ascites and hydrothorax. Fetuses with hydrops fetalis may die in utero from profound anemia and circulatory failure. If they survive the initial birth process, they develop hyperbilirubinemia as the result of the inability of the liver to conjugate the bilirubin being produced by excessive destruction of red cells. The end point of the untreated disease in a surviving infant is the production of kernicterus with deposition of bile in the basal ganglia of the neonatal brain. Fetal renal function appears to be inadequate in that the fetus cannot excrete the excessive fluid and does not respond to normal diuretic administration.

The majority of infants with hydrops fetalis and the profound circulatory problems which accompany it have a readily identifiable immune factor to account for it. However, there is a group of nonimmune cases of hydrops fetalis which demonstrates all of the clinical signs of the immune group, but the group of nonimmune cases has no identifiable circulating antibodies or other disease process to account for the symptoms. This group presents an interesting problem, and our recent experience in South Florida has been that, although these cases occur rarely, the women who produce these babies have all come from rural areas and have been involved in agricultural activities. This may or may not have any significance in the etiology. It is, however, a reasonable question to ask whether agents which are commonly used in agriculture can induce hydropic changes in the fetus.

Disorders of Growth and Function in Multiple Gestation

Mortality and morbidity are markedly increased in twin gestation. The most profound cause of mortality at the present time is prematurity; however, there are other risks associated with twin pregnancy because of the possibility that the circulation of the twins may be interrelated before birth. Twins may be either monozygous (developed from a single ovum) or dizygous (each having a separate ovum). The placenta in twin pregnancies may be conjoined placenta when the possibility exists of a common circulation prior to birth. This is particularly true in monochorionic placenta where the chorionic membrane encloses both sacs. A recent study by our group has demonstrated an extremely high risk of placental anastomosis in patients who have monochorionic placentas, and these vascular communications may allow gross admixture of blood between the fetuses (Robertson, Neer 1983). Shifts of blood between the fetuses may then lead to significant hypervolemia and hyperviscosity in the recipient and hypovolemia and anemia in the donor. This is described as the twin-twin transfusion syndrome. In this case one twin may be very much smaller than the other as a consequence of chronic intrauterine malnutrition. The anatomic changes in the underperfused twin resemble those found in growth-retarded singletons where the placentas were extensively infarcted (Naeye 1965). The differences in hemoglobin concentration between the two twins may be profound. Hypotension, microcardia, and generalized growth retardation characterize the overtly affected hypovolemic twin, in contrast to hypertension and cardiac hypertrophy in the hypertransfused twin. Polyhydramnios, perhaps as a consequence of increased renal perfusion and increased urine formation, may accompany the hypervolemia and polycythemia of the larger infant. In this situation both fetuses are at risk, and the underperfused twin may die in utero; the extreme situation produces a fetus papyraceous where the dead fetus is reabsorbed. Dangerous circulatory overload with heart failure may complicate the neonatal period if severe hypervolemia and blood hyperviscosity at birth are not promptly identified and treated. The risk of this occurrence, however, is very low in spite of the high incidence of placental anastomosis.

A further risk in twin pregnancy is that of chimerism where there is a mixture of genotypes in the twin pregnancy from more than one ovum and sperm (Bernirschke 1974). This can occur only when there is cross circulation between the placentas in a dizygotic twin pregnancy, and this was demonstrated on one single occasion in our own study. Cells are transfused from one similar fetus to another, and these transfused cells are not destroyed since exposure of the recipient twin to the dissimilar antigens of the donor twin early in fetal development renders the recipient twin tolerant to the donor twin tissues.

Although most twin fetuses do not suffer from severe risks, a minority have severe problems with functional development. Consequently, they require careful evaluation following delivery because of the risk that this poses to their subsequent development.

Fetal Cardiac Arrhythmias

A major functional cardiac problem which is seen infrequently is that of the prenatal detection of a gross cardiac arrhythmia. Little is known of the etiology or the significance of these arrhythmias, but when detected they cause the obstetrician considerable concern. Those that have been seen have taken a form usually of an irregular fetal heart pulse, and it is only recently that it has become possible to perform studies on the intact fetus prior to birth. The use of an abdominal processor with a fetal cardiac monitor enables a fetal electrocardiogram to be displayed even when the membranes are intact. Fetal echocardiography, a relatively new technique, has been used recently to study these patients who have infants with arrhythmias. In some cases the precipitating cause is known. For example, in our unit a mother who presented at 35 weeks of gestation and whose fetus had a grossly irregular arrhythmia, had recently had a severe viral infection. The assumption was that the fetus had contracted a viral myocarditis. External electrocardiography was attempted but without success. An echocardiogram performed by ultrasonography showed dilatation of the ventricular system of the heart, suggesting that the fetus was in incipient heart failure. The mother was admitted to hospital and was given a small dose of digitalis. Over the course of the next 2 weeks the fetal heart became more regular. She went into labor spontaneously at 37 weeks and, although the infant at birth still had an irregular heart rate, this condition responded to standard cardiac therapy and the fetus subsequently made satisfactory recovery. Other cases, however, have been seen where the initiating insult to the myocardium was not known. In these cases the management has been extremely difficult in that we do not know whether it is safer to continue to treat the fetus _in utero_, where it is possibly still exposed to an environmental toxin, or whether the baby should be delivered and be treated in the neonatal intensive care unit. Both approaches have been adopted, and usually these arrhythmias turn out to be benign and disappear within 24 to 48 h of delivery. Since there are many agents which can induce cardiac arrhythmias in the environmental field, it would be interesting to follow up these infants. Unfortunately, a single center (even a large tertiary-level care unit) will not see very many of these cases. We see approximately two cases per year, and usually the stress of the clinical decision-making process tends to lead us away from investigatory procedures that might lead to a better understanding of the significance of this functional defect.

CONCLUSIONS

The most serious problems in obstetrics and neonatology still remain the problems of prematurity and congenital structural defects. However, there is an increasing number of fetuses and infants who are seen with functional defects when the fetus is mature. These constitute severe risk problems to the growth and development of the baby and require an increasing amount of attention, both from the point of view of research and for development of clinical protocols for management.

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ASSESSMENT OF THE UTILITY OF POSTNATAL TESTING FOLLOWING PRENATAL EXPOSURE TO FORTY CHEMICALS

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INTRODUCTION

Although it has been widely recognized since the 18th century that the developing organism is uniquely susceptible to some chemicals and diseases, the science of developmental toxicology is in its infancy. Presently, it is recognized that chemicals should be tested for their ability to produce defects during the prenatal period. This awareness arose as a consequence of the thalidomide-induced birth defects in children in the 1950's. After this incident protocols were developed to examine the potential of a chemical to cause abnormalities in experimental animals (World Health Organization 1967). In these teratology studies, pregnant animals, usually rodents, are exposed to the chemical, and their fetuses are examined near term for skeletal and soft-tissue defects. Limiting the teratologic examination to a gross morphological evaluation of the fetus is of concern, because it is becoming increasingly apparent that prenatal exposure to chemicals can result in functional deficits without producing frank terata. Such effects have been observed in studies using rodents which have evaluated postnatal behavioral (Vorhees et al. 1979; Olson et al.

1980a,b; Osborne et al. 1980; Nelson et al. 1980, 1981; Abel 1980; Schalock et al. 1981; Gray et al. 1981; Rodier 1976; Zagon et al. 1979), reproductive (Walker 1981; McLachlan et al. 1975), and kidney development (Kavlock, Gray J 1982; Kavlock, Gray LE 1983). In addition, the morphological developmental landmarks, such as eye opening, incisor eruption, and vaginal opening, which are regulated by diverse physiological processes, can also be altered by prenatal exposure to a chemical. Similar abnormalities of development have been observed in humans following *in utero* exposure to xenobiotic chemicals. Reproductive tract lesions and reduced reproductive ability have been noted in men and women exposed *in utero* to diethylstilbestrol (Schmidt et al. 1980), behavioral alterations occur in children of heroin addicts and alcoholics (Hutchings 1978), and fetal methyl mercury exposure alters the development of the brain and behavior (Chang 1977).

The present report is an evaluation of the utility of observing postnatal growth, viability, morphology, locomotor activity, and reproductive function following prenatal exposure to chemicals. We have developed a protocol to evaluate a large number of chemicals, some of which are known teratogens in mice, known nonteratogens, and unknowns. The protocol used in the first experiment is shown in Fig. 1; subsequent studies employed similar methodologies. The chemicals and dosing periods are shown in Table 1. The objectives of this experiment were to determine if our postnatal testing provided any essential information about the teratogenic potential of chemicals. We wanted to determine if the survivors from teratogens were normal; if the growth retardations seen at birth were persistent or transient; if postnatal mortality extended past the early neonatal period following exposure to teratogens; or if nonteratogenic, nonfetotoxic chemicals produced alterations of growth, viability, morphology, behavior, or fertility. After a number of treatment conditions were tested, the protocol was modified and additional chemicals were tested. The results of our studies of Nitrofen, a classic postnatal teratogen, using three rodent species, are discussed, because they clearly demonstrate the utility of postnatal testing.

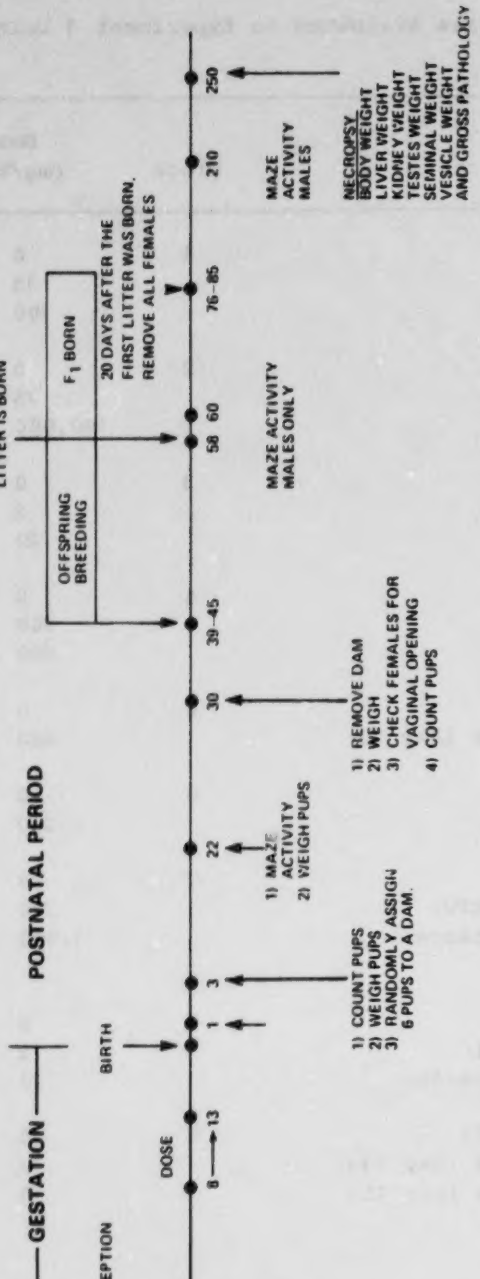

GESTATION
POSTNATAL PERIOD
CONCEPTION
DOSE
BIRTH
0
8 → 13
1
3
22
30
39–45
58
60
76–85
210
250
1) COUNT PUPS
2) WEIGH PUPS
3) RANDOMLY ASSIGN 6 PUPS TO A DAM.
1) MAZE ACTIVITY
2) WEIGH PUPS
1) REMOVE DAM
2) WEIGH
3) CHECK FEMALES FOR VAGINAL OPENING
4) COUNT PUPS
OFFSPRING BREEDING
ISOLATE THE FEMALES JUST BEFORE THE FIRST LITTER IS BORN
F1 BORN
20 DAYS AFTER THE FIRST LITTER WAS BORN, REMOVE ALL FEMALES
MAZE ACTIVITY MALES ONLY
MAZE ACTIVITY MALES
NECROPSY
BODY WEIGHT
LIVER WEIGHT
KIDNEY WEIGHT
TESTES WEIGHT
SEMINAL WEIGHT
VESICLE WEIGHT
AND GROSS PATHOLOGY

Fig. 1. Postnatal mouse teratology protocol.

Table 1. Chemicals Evaluated in Experiment 1 Using CD-1 Mice

Chemical	Block	Dose (mg/kg/d)	
Control	1	0	(oral)
Dinoseb		15	
Carbaryl		100	
Control	2	0	
Toxaphene		75	
Vitamin A (IU/kg)		160,000	
Control	3	0	(oral)
Endrin		2	
Chlordecone		20	
Control	4	0	(oral)
Caffeine		200	
Cacodylic acid		600	
Control (Day 8)	5	0	(ip)
5-Bromodeoxyuridine (Day 8)		400	
Control	6	0	(oral)
Benomyl		200	
Control	7	0	(oral)
Ethylenethiourea (ETU)		300	
Ethylenediaminetetraacetic acid (EDTA)		1,000	
Control	8	0	(oral)
Endrin (Days 14-18)		2	
Chlordecone (Days 14-18)		20	
Control (Days 11,15)	9	0	(ip)
6-Aminonicotinamide (Day 11)		10	
6-Aminonicotinamide (Day 15)		10	

(continued)

Table 1. (continued)

Chemical	Block	Dose (mg/kg/d)	
Control	10	0	(oral)
Decamethrin		10	
Ethylenebisisothiocyanate (EBIS)		200	
Control	11	0	(oral)
Hexachlorocyclopentadiene		45	
Cyclohexanone		800	
Control	12	0	(oral)
Chlordecone		20	
1,2,4-Trichlorobenzene		130	
Control	13	0	(oral)
Chlordane		50	
Lindane		25	
Control	14	0	(oral)
Mirex		7.5	
Thalidomide		400	
Control	15	0	(oral)
2,4,5-Trichlorophenoxyacetic acid		130	
Nitrofen		200	
Control	16	0	(oral)
Sodium Salicylate		800	
Lithium Carbonate		400	
Congo Red		1000	
Control	17	0	(oral)
Kelevan		125	
Nickel Chloride		100	
Control (Day 8)	18	0	(ip)
Nickel Chloride (Day 8)		30	
Diazo Red RC (Day 8)		150	
Alcian Blue (Day 8)		250	

(continued)

Table 1. (continued)

Chemical	Block	Dose (mg/kg/d)	
Control (Day 8)	19	0	(ip)
Congo Red (Day 8)		300	
Control (Days 11,12,14,15)	20	0	(ip)
Aminopterin (Day 11)		20	(ip)
Cortisone (Day 12)		300	(im)
Cytosine Arabinoside (Days 14/15)		30	(ip)
Control	21	0	(oral)
Thalidomide		2,000	
2,4,5-Trichlorophenol		800	
Sodium Selenite		10	

METHOD OF EXPERIMENT 1

In the first experiment pregnant mice were exposed to 36 different chemicals in 42 different treatment combinations (Table 1). Treatments were administered by gavage on Days 8 through 12 of pregnancy unless otherwise noted in Table 1. The dams were allowed to deliver; and the postnatal viability, growth, morphology, locomotor activity, and reproductive function of their offspring were assessed. The study was conducted in 21 blocks with 2 or 3 treatment groups and a concurrent control per block. Within each block, each treatment group initially contained 23 to 40 primiparous 60-d-old female mice (CD-1). Doses were selected either from previously published studies, or from a preliminary study conducted in order to determine a dose that would be minimally toxic to the dam. The females were allowed to give birth, and the litters were counted and weighed at 1 and 3 d of age. Dead pups recovered from the cages were necropsied and abnormalities recorded. Dams that had not given birth by Day 3 were killed and examined for the presence of resorptions. Litter sizes were reduced to a uniform number in order to reduce the variability in growth rates of the pups. Four to twelve dams were randomly selected on postnatal Day 6 from each treatment group and given six pups (three males and three females) which were randomly selected from the entire population of pups from all the dams in that treatment group.

At 21 d of age the figure eight maze locomotor activity levels of a male and a female from each litter were observed for 60 min during the diurnal portion of their activity cycle. Figure eight maze reactive locomotor activity was selected because we have found that this procedure is sensitive to behavioral alterations induced in this species by subchronic treatment with lead (Gray, Reiter 1977) and manganese (Gray, Laskey 1980), and by acute exposure to endrin (Kavlock et al. 1981) or amphetamine (Gray, unpublished data). Similar mazes have also been used extensively for studies using rats (Reiter et al. 1977; Wong, Klassen 1980; Buelke-Sam et al. 1982). Each maze contains eight photocells, and, as an animal ambulates, the number of beam interruptions occurring in a 1-h period is recorded. These observations were repeated at 58 and 210 d of age. Females were not tested at this time because many were pregnant. At 30 d of age the dams were removed, and the number of males and females per litter, their weights, and the number of females with patent vaginas were determined. Throughout the remainder of the experiment the animals were checked while the cages were being changed, and obviously pregnant females were removed and individually housed until parturition, at which time the litter size and the age of the dam were recorded. After the final observation of reactive locomotor activity in the maze, at approximately 250 d of age, the males were necropsied; and body, liver, testes, seminal vesicle, and right kidney weights were taken. The organ weights recorded at this time reflect our interest in reproductive and kidney physiology and should not be construed as a comprehensive gross pathological analysis; however, any obvious gross pathology seen at this time, or at any other time in the experiment, was recorded.

In blocks 20 and 21, the postweaning protocol was modified to improve the sensitivity of our tests and shorten the length of the experiment. At 35 d of age a single male was housed with a single non-littermate female rather than housing three males and three females together. This was done for two reasons: 1) the data from block 16, in which 25% of the Congo red males had atrophic testes, demonstrated that this was an insensitive measure of male fertility, because only one of the three males needed to be fertile to impregnate 100% of the females; and 2) the male survival after they began breeding was poor due to occasionally high levels of intermale aggressive interactions. In addition, in these last two blocks maze activity was measured only at 21 d of age and the males were necropsied at 150 d of age.

RESULTS OF EXPERIMENT 1

The effects of prenatal administration of the 36 chemicals, administered as 42 different treatments, on postnatal viability, body weights on Days 1 and 3, and maternal viability and weight change during dosing are presented in Chernoff and Kavlock (1982, 1983).

The analysis of the control data indicated that there were significant block effects for all parameters measured in the present experiment, including growth, viability, reproductive performance, and locomotor activity, indicating that treatments could be compared only with the concurrent control group. Similar block effects were observed by Chernoff and Kavlock (1982, 1983) on maternal weight, maternal weight gain, litter sizes, and pup weight on Days 1 and 3 in the first phase of this experiment; and Butcher (1981) reported large interlaboratory differences between five laboratories using uniform behavioral test procedures.

Seventeen of the forty-two treatment conditions employed in the first experiment reduced the numbers of live pups on Day 3 by 10% or more, indicating that they were fetotoxic or teratogenic (Table 2). Subsequent observations of viability indicated that continued survivorship was not correlated with the number of live pups on Day 3 (Table 2). Of the 17 chemicals that reduced litter sizes on Day 3, only benomyl and Nitrofen caused additional offspring mortality between 3 and 30 d of age; and cytosine arabinoside (ip, Day 14 and 15 of gestation), which caused only an 18% reduction in viability on Day 3 and did not increase mortality between 3 and 30 d of age, produced a 53% decrease in viability after weaning.

The prenatal administration of both Nitrofen and cytosine arabinoside caused the development of serious abnormalities, some of which were incompatible with life,

Table 2. Effects of Prenatal Exposure to Chemicals on the Postnatal Viability of Offspring[a]

	Relative Mortality Due to Treatment[b] (%)		
Treatment	Day 3	Day 3 to 30	Day 30 to 60
Aminopterin	92	-31	0
Cortisone	86	-12	12
Nitrofen	82	21	-4
Benomyl	63	23	15
Alcian Blue	62	0	0
Mirex	56	-11	0
6-Aminonicotinamide (Day 11)	53	-5	-5
Bromodeoxyuridine	51	9	0
Vitamin A	41	10	17
2,4,5-Trichlorophenoxyacetic Acid	34	0	-4
Nickel Chloride (ip)	32	10	0
Congo Red (ip)	30	0	0
6-Aminonicotinamide (Day 15)	29	2	15
Cacodylic Acid	24	-5	-14
Chlordecone (Days 8 through 12) (blocks 3 and 12)	21	-2	7
Cytosine Arabinoside	18	-12	53
Caffeine	18	4	0

[a] The data presented include those for chemicals which reduced viability by more than 10% on Day 3. The data demonstrate the lack of correlation between Day 3 survivorship and subsequent viability.

[b] Control-treated/control x 100.

that could not be detected in the neonates. Some of the Nitrofen-treated pups became hydrocephalic and died before weaning, and many of the cytosine arabinoside pups did not have lower incisors and were also hydrocephalic and died. In addition to these defects, further abnormalities were observed in some of the surviving offspring. Survivors of prenatal exposure to lethal chemicals like benomyl, 6-aminonicotinamide (ip, Day 11), cortisone (im, Day 12), and Nitrofen were not normal. However, the survivors of other treatments like 5-bromodeoxyuridine (ip, Day 8) and 2,4,5-trichlorophenoxyacetic acid were normal.

The lack of correlation between the reductions in body weight on Days 1 and 3 with the subsequent growth of the offspring exposed prenatally to chemicals is even more striking than the inability to predict viability from Day 3 litter size. Some of the Day 3 growth reductions were transient, while others persisted, and in two cases the effects became more severe (Table 3). For example, diazo red (ip, Day 8) produced a 15% reduction in Day 3 weight, but subsequent growth was normal; and similar effects were noted with chlordecone (Days 8 through 12), mirex (17% reduction on Day 3), and sodium salicylate (10% reduction on Day 3). The 30% weight reduction noted in the 6-aminonicotinamide (ip, Day 15) offspring on Day 3, the largest weight reduction seen on Day 3 in any of the chemicals tested, was attenuated to 13% by Day 22. Conversely, Day 3 growth retardations induced by cortisone, cacodylic acid, Nitrofen, and toxaphene persisted; and treatment with cytosine arabinoside caused a reduction in body weight of 11% at Day 3 which increased to 40% by Day 22. In addition, the offspring exposed to aminopterin (ip, Day 11) were growth retarded throughout the experiment, although Day 3 weight was 9% heavier than concurrent controls.

The MANOVA and subsequent ANOVA indicated that 9 of the 42 treatment conditions caused permanent reductions in body weight. These treatments included chemicals that did not reduce Day 3 weight, such as Vitamin A and aminopterin, and those that did: cytosine arabinoside, cortisone, Nitrofen, 6-aminonicotinamide (Day 11), and cacodylic acid. Alcian blue and toxaphene produced similar weight reductions both at necropsy and at 3 d of age, but the effects were significant only at necropsy. Of the treatments which reduced body weight, five of them also reduced liver weight, three reduced seminal vesicle weights, and two lowered kidney weights at necropsy. In addition, chlordecone

Table 3. Effects of Prenatal Exposure on the Postnatal Growth of Offspring[a]

Treatment	Relative Body Weight[b] (%) Day 3	Day 30	Day 210
6-Aminonicotinamide (Day 15)	-30	-9	-10
Nitrofen	-24	-16	-12
6-Aminonicotinamide (Day 11)	-20	-10	-12
Cortisone	-18	-16	-20
Mirex	-17	-8	-3
Cacodylic Acid	-16	-4	-12
Diazo Red RC	-15	-5	0
Chlordecone (Days 8 through 12)	-13	-3	+2
Cytosine Arabinoside	-11	-42	-21
Sodium Salicylate	-10	-1	+4
Caffeine	-9	-5	-5
Congo Red (oral)	-9	-3	-7
Toxaphene	-9	-5	-7
Alcian Blue	-8	-15	-9
Benomyl	-4	-18	-3
Vitamin A	-3	-4	-12
Aminopterin	+9	-7	-10

[a] The data are for those chemicals which retarded growth at some point in the experiment. The data are presented as the weight of the concurrent control minus the weight of the treated group divided by the concurrent control group. A correlation analysis of the data indicated that Day 3 weight was not correlated with weight at any of the subsequent ages.

[b] Control-treated/control x 100.

(Days 8 through 12 of gestation) reduced kidney weights in blocks 3 and 12, as did mirex in block 14, without affecting body weights. It is important to note that the effect of chlordecone (Days 8 through 12 of gestation) on kidney weights at necropsy were replicated even though the blocks differed significantly in necropsy weights.

The gestational administration of 6-aminonicotinamide on Day 11 reduced the fertility rate by 34% and appeared to accelerate the age at parturition. Perhaps those females which were going to breed were quickly inseminated by the males, and those that would have been bred later did not cycle at all. The reproductive organ weights of the males in this treatment group were less than controls, as was body weight, but this difference was not sufficient to suggest that the males were sterile.

The oral administration of Congo red on Days 8 through 12 of gestation produced a 30% reduction in testes weights in the offspring, and it delayed breeding and reduced litter sizes ($p = 0.003$, MANOVA). The use of the mean testes weight for this group is somewhat misleading, as it was clear that the treated males fell into two distinct populations. Most of the males had normal testes, weighing about 290 mg, while the other 25% had testes weighing less than 100 mg. The ip administration of Congo Red on Day 8 of gestation was less effective than the repeated oral dosing, but it too reduced testis weight. The oral administration of this chemical was not teratogenic or fetotoxic as indicated by the number of live pups on Days 1 or 3, whereas the injection of Congo red resulted in resorptions in 4 of the 15 pregnant females.

Seven of the forty-two treatments in the first experiment altered reactive locomotor activity levels in the mazes. Prenatal administration of three of these chemicals caused hyperactivity at Day 22 (chlordane, Nitrofen, and cytosine arabinoside). Cytosine arabinoside produced the most dramatic effect, increasing locomotor activity levels by nearly 100% of control (Fig. 2). The effect of chlordane was transient, whereas the hyperactive Nitrofen offspring died shortly after testing as their hydrocephalic condition became more severe (Fig. 3, middle left). The cytosine arabinoside pups were not retested. Three chemicals administered during gestation resulted in hypoactivity in the offspring at 22 d of age but had no effect on later activity levels (chlordecone (Days 8 through 12), 6-aminonicotinamide, and decamethrin). The

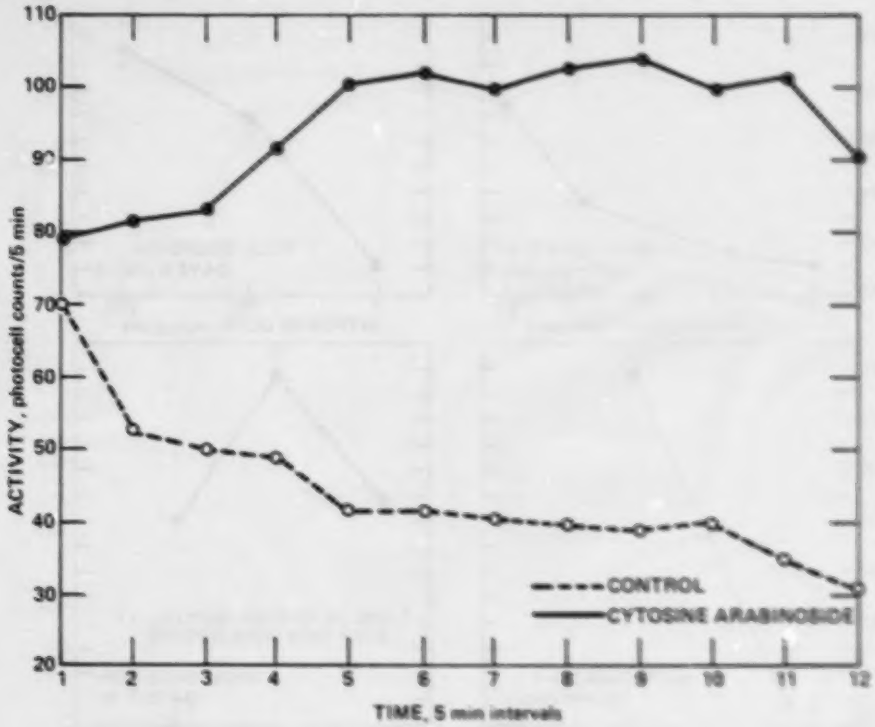
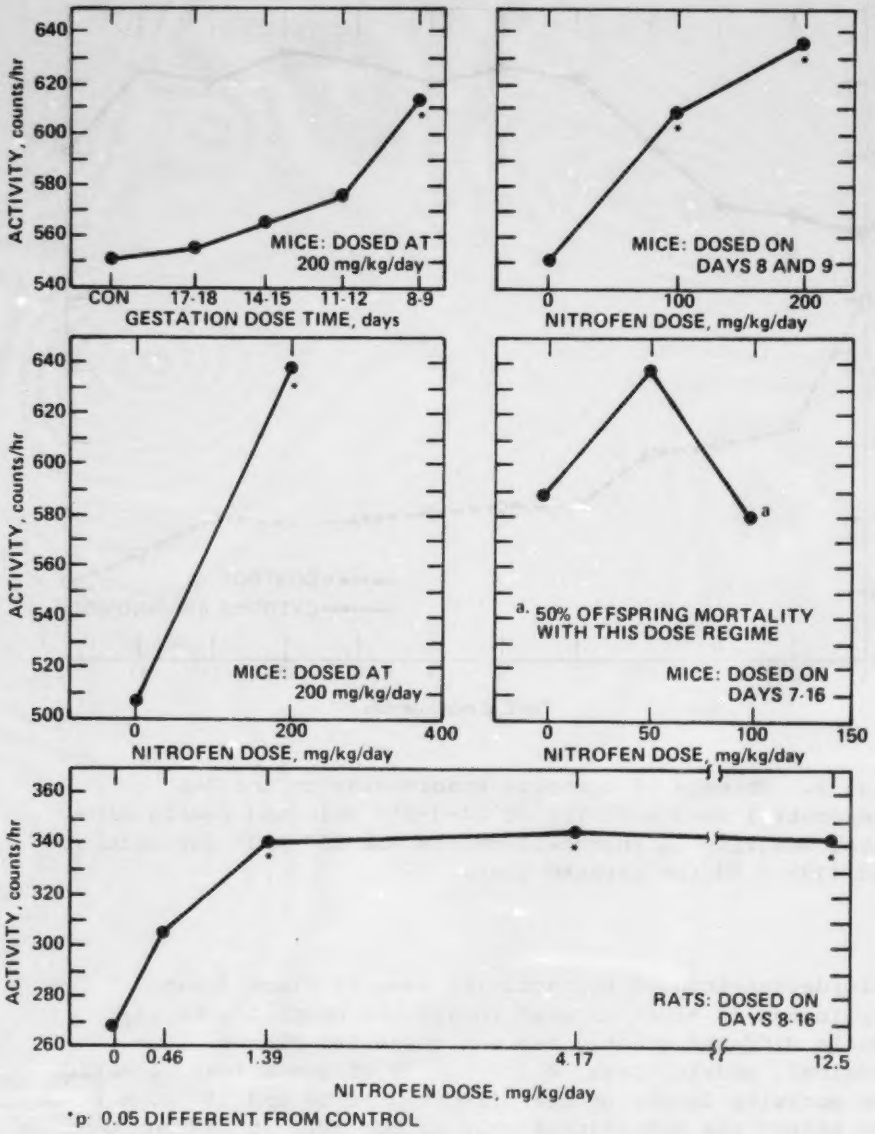


Fig. 2. Effects of cytosine arabinoside on the 1-h residential maze activity of 21-d-old male and female mice. Total activity in photocell counts was 557 ± 31 for control and 1137 ± 58 for treated mice.

chlordecone-induced hypoactivity seen in block 3 was replicated in block 12 even though the control activity levels differed greatly between these two blocks. One chemical, endrin (Days 14 through 18 of gestation), lowered the activity levels of the offspring at 58 and 210 d, but the effect was significant only at Day 58. It is important to note that for endrin (Days 14 through 18 of gestation), decamethrin, and chlordane no effects other than these alterations of locomotor activity were seen. Such alterations of locomotor activity warrant replication and additional study.



*p 0.05 DIFFERENT FROM CONTROL

Fig. 3. Effects of in utero exposure to Nitrofen on the figure eight maze locomotor activity levels in weanling Sprague-Dawley rats and CD-1 mice.

NITROFEN TERATOLOGY EXPERIMENTS IN MICE

The effects of Nitrofen on the Harderian gland and activity seen in the first experiment represent the type of effects that cannot be adequately assessed using standard teratology protocols. Since the initial observations of Nitrofen in Experiment 1, our laboratory has participated in over 15 additional studies using Nitrofen. These studies have utilized both chronic and acute dosing regimes during gestation and have been conducted in rats, mice, and hamsters. In a standard teratology test, prenatal exposure to Nitrofen at 200 and 100 mg/kg/d on Days 7 through 16 of gestation produced gross morphological defects in only 12% and 3% of the fetuses, but 100% and 50% of the pups from those dams allowed to litter at this dose died within 72 h of birth (Table 4). In addition, the abnormalities observed did not include microphthalmia, anophthalmia, or hydrocephaly, effects which were seen at weaning. Many of the pups at the higher doses developed distended abdomens from gasping air, failed to suckle, and died. Other abnormalities noted in the fetuses and pups included cleft palate, diaphragmatic hernia, and undescended testes. Although Nitrofen induced few gross morphological abnormalities in the fetuses and pups, the survivors subsequently developed many other alterations, including growth retardations following doses which resulted in normal body weights at birth; Harderian gland destruction at 25 mg/kg and above; and lung, liver, and seminal vesicle weight reductions; and reduced fertility (Gray *et al*. 1982a, 1983).

In another study using mice a "pulse" dosing regime was used. Dams were dosed with a chemical on either Days 8 and 9, 11 and 12, 14 and 15, or 17 and 18 of gestation; and the postnatal development of their offspring was followed as in the last two blocks of Experiment 1. The results of the effects on Nitrofen on maze activity (100 and 200 mg/kg/d), are presented in Fig. 3 (upper left and right). Exposure to Nitrofen at 100 or 200 mg/kg/d on Days 8 and 9 of gestation produced a dose-related hyperactivity in the 22-d-old offspring. In the offspring which received Nitrofen at 200 mg/kg/d on Days 8 and 9 many defects developed. At 15 d of age 15% of the pups were hydrocephalic, and most of these died before the behavioral testing, and 92% of the animals had no Harderian glands. This effect on the Harderian gland caused the eyes to appear small in some of the weanling

Table 4. Perinatal Effects of Nitrofen Administered Orally on Days 7 to 17 of Pregnancy in the Mouse (CD-1).

Effect	Dose of Nitrofen (mg/kg/d)			
	0	50	100	200
Fetal Observations				
Number of implants	10.2	10.4	11.0	10.5
Mortality (%)	9.7	5.5	5.4	17.0
Weight (g)	0.97	0.97	0.95	0.95
Total defects[a] (%)	2/17	1/13	3/19	12/46
Neonatal Observations				
Number live: Day 1	10.7	10.6	9.6	7.6[b]
Day 3	10.4	8.7[b]	4.2[b]	0.2[b]
Pup weight (g): Day 1	1.62	1.51[b]	1.40[b]	1.33[b]
Day 3	2.02	1.85[b]	1.53[b]	1.18
Number of dead pups recovered and examined	6	26	50	72
Defects in the Dead Pups				
Number with cleft palate	1	1	1	11
Number with diaphragmatic hernia	0	0	0	4
Number with distended abdomens	0	2	12	16
Total number affected	1	3	13	31

[a] Values are (% of individuals affected/% of litters).

[b] Differs significantly from the control value.

mice, while the eyes were shut in the rest of the treated offspring. After weaning, growth rates and viability were reduced and some of the mice developed malocclusion of the jaws. Similarly, 50% of the offspring lacked Harderian glands in the 200 mg/kg/d on Days 11-12 treatment group. In the 100 mg/kg/d on Days 8-9 treatment group the animals appeared grossly normal, although they were hyperactive; and at necropsy, lung, testis, and Harderian gland weights were depressed in the absence of any effect on body weight.

NITROFEN TERATOLOGY EXPERIMENTS IN RATS

Similar studies with Nitrofen using rats indicate that acute and chronic dosing causes hydronephrosis (Kavlock, Gray 1983) and, at lower doses, alterations of kidney function (Kavlock et al. 1982). Chronic gestational dosing causes a dose-related hyperactivity (Fig. 3) at doses above 0.46 mg/kg/d on Days 8 through 16, reduced Harderian gland weight at 4.17 mg/kg/d and above, and diaphragmatic hernias at doses above 0.46 mg/kg/d (Ostby et al. 1983). In a study using Fischer 344 rats, in which the dams received Nitrofen at higher doses for a shorter duration, we have observed that lung weights were persistently affected in the female offspring exposed to Nitrofen at 15 mg/kg/d on Days 10 through 13 of gestation. The female offspring were necropsied at 50 d of age, and the control body, lung, and Harderian gland weights were 125.6 g (±1.9), 807.5 mg (±31), and 172.1 mg (±1.9), respectively; whereas in the Nitrofen-treated group the values were 120.6 g (±2.2), 682.3 mg (±15, $p < 0.01$), and 115.3 mg (±2.1, $p < 0.0001$). In a second experiment using Fischer 344 rats and a dosing protocol similar to the first, body and organ weights were lowered by treatment with 20 mg/kg/d, whereas only Harderian glands were significantly smaller at 10 mg/kg/d. Interestingly, 3 of the 21 rats in the 20-mg/kg/d group had diaphragmatic hernias when necropsied after weaning, as did 1 of 26 at 10 mg/kg/d. In addition, one male had an undescended testis and one female was missing a right uterine horn; both animals were in the 20-mg group. In a study using Long Evans rats, the dams were dosed with 75 mg/kg on Day 11 of gestation and the development of their offspring observed (Kavlock, Gray 1983). Transient lung weight effects, persistent hydronephrosis, altered kidney function, enlarged testes, and reduced Harderian gland weights (10% missing) were noted, while body, liver, seminal vesicle, and thyroid weights were not affected. In a similar study using Sprague-Dawley rats, the dams were dosed with 50 or 100 mg/kg/d of Nitrofen on Day 11 of gestation.

In this study 15% of the low-dose and 50% of the high-dose offspring lacked Harderian glands. In summary, in the rat, chronic dosing with very low doses of Nitrofen causes diaphragmatic hernias (at 0.15 and 1.39 mg/kg/d) and hyperactivity (at 1.39 mg/kg/d) and alters kidney function; at higher doses, chronic dosing reduces Harderian gland weight; whereas acute dosing on Day 11 of gestation or dosing on Days 10 through 13 alters kidney function and affects the Harderian gland at doses below those at which other organ weights are affected.

NITROFEN TERATOLOGY EXPERIMENT IN HAMSTERS

In other studies using hamsters, dams were pulse-dosed on Days 8-9, 11-12, or 14-15 of gestation with Nitrofen at 400 mg/kg/d. This dosing regime does not reduce neonatal viability or growth. As in rats and mice, lung and Harderian gland weights were specifically affected by Nitrofen treatment, with the 8-9-d regime being the most effective. Growth, not affected at birth, was subsequently retarded by treatment at the three stages of gestation. The reproductive system of the hamster was severely affected, and the results indicated 1) that Nitrofen was interfering with meso-, meta-, and paramesonephric duct development, as indicated in the females by the presence of uterus unicornis, often occurring with ipsilateral renal agenesis and 2) that it was causing alterations of seminal vesicle gland development, a mesonephric duct derivative, in the males. In the second experiment using hamsters the dams were dosed with Nitrofen at doses ranging from 25 to 400 mg/kg/d on Days 7 through 11 of gestation. Reproductive development was affected at all doses, as indicated by reduced size of the testosterone-dependent flank gland of the male. In addition, the seminal vesicle position in the body cavity was abnormal, and weights were reduced at all doses (significantly at 100 mg and above), representing a subtle alteration of mesonephric duct development.

DISCUSSION

The results of the present study clearly demonstrate that prenatal exposure to chemicals can cause abnormal postnatal development and that many of the abnormalities cannot be predicted from a careful examination of the newborn pup. It is possible for such serious effects to escape detection using neonates because of their undeveloped condition. Those systems that differentiate prenatally but do not function until postnatal life cannot be comprehensively evaluated in immature animals. A few of the susceptible systems include the reproductive system, the central nervous system, and the endocrine system. In the present study we found that some prenatal treatments (Nitrofen and cytosine arabinoside) which only slightly reduced litter sizes at 3 d of age caused extensive mortality at or near weaning, whereas other treatments resulted in persistent growth retardations that could not be anticipated from the neonatal data. Central nervous system function, as measured by locomotor activity, and reproductive function were specifically affected by some prenatal treatments. Other chemicals used in the present study produced a variety of unusual developmental abnormalities, including missing eyes, Harderian glands, and teeth; hyperactivity and hydrocephaly; and malformed seminal vesicles.

The pups prenatally exposed to cytosine arabinoside, a known central nervous system teratogen, were nearly twice as active as the controls were in the mazes at 22 d of age. Furthermore, they became hydrocephalic late in the lactation period, and many of them (30%) lacked lower incisors. Two of these pups had no incisors at all and 50% of them died after weaning. None of these effects were detectable in the newborn and only the hydrocephaly would be detectable in an examination of the fetus.

The other chemical which produced a variety of unusual postnatal alterations was Nitrofen. The mice and rats prenatally treated with Nitrofen were also hyperactive, a few of the mice at the highest doses were hydrocephalic, and some of these pups died. In addition, the age of eye opening was delayed in mice, and a few of the mice had no eyes at all. We have demonstrated that the apparent anophthalmia or microphthalmia in rats and mice prenatally exposed to Nitrofen cannot be detected in the fetus or neonate and arises as a result of the destruction of the Harderian gland in the orbit. This gland is small and does not proliferate until the second week of life, and the absence of this gland or its small size in other animals causes the orbital defects, delaying eye opening in this species. In a study using mice exposed prenatally to

Nitrofen at 200 mg/kg/d on Days 7 through 16 of gestation, all of the offspring died after birth even though a teratology study indicated that only 12% of them were abnormal. In studies using rats, Kavlock and Gray (1983) have seen that postnatal kidney function is altered by prenatal Nitrofen administration, and in our studies using hamsters we have found that this chemical causes reproductive tract lesions that are apparent only in adults (Gray et al. 1982b).

It is of particular interest that among the treatments that failed to reduce neonatal survival or growth, or produce obvious morphological abnormalities (Chernoff, Kavlock 1982, 1983), one produced severe alterations of testicular development, and three of the chemicals significantly affected locomotor activity in the present postnatal study. These chemicals would appear to be nonteratogenic following standard teratology bioassay studies in mice. Oral administration of the diazo dye Congo red during Days 8 through 12 of gestation or ip injection on Day 8 was teratogenic to the reproductive system of the male. Subsequent studies in our laboratory have demonstrated that prenatal exposure to Congo red reduces the gametogenic potential of the male but does not affect testosterone production or the sex accessory glands.

CONCLUSIONS

The present study was designed to determine the utility of postnatal testing following prenatal exposure to chemicals, to prioritize chemicals for additional studies, and to identify some of the practical problems encountered in doing such a large study. Even with the limited methodology employed, we have clearly demonstrated that useful information concerning the teratogenic potential of a chemical can be obtained in a postnatal study like the present study. Certainly the teratogenic potential of Nitrofen, cytosine arabinoside, and Congo red have been more clearly defined by the present study.

There are other basic experimental problems that result from trying to rapidly "screen" a large number of chemicals as in the present study. From a statistical point of view, we have measured so many independent variables that if we had used t-tests to analyze the data the probability of a

Type I error, rejecting the null hypothesis when it is true, would be very high. On the other hand, had we adjusted the t-test using the Bonferroni approach (Harris 1975), which adjusts the critical values for the number of comparisons made between each treatment and the concurrent control, we would have not found that the Nitrofen pups were hyperactive, that chlordecone produced hypoactivity in block 3, and that Congo red males had smaller testes, all effects that have been replicated. Our solution to this problem is to repeat those treatments which produce an effect. Another statistical solution would be to reduce the number of measurements made; however, a neurotoxicologist or a kidney physiologist could claim (correctly so) that we have not made enough measurements to properly assess the function of those systems and that other systems were not examined at all. Our postnatal evaluation of many systems is dependent upon the assumption that they are essential for normal growth and viability. For example, we would expect that severe alterations of thyroid, adrenal, and pituitary function or that altered kidney, lung, cardiac, or other organ physiology or morphology would retard growth and reduce postnatal viability.

In addition to these studies emphasizing prenatally induced developmental abnormalities, we have conducted other studies in which we dosed pups during the neonatal period, because some prenatal developmental events in humans occur during the early neonatal period in rats, mice, and hamsters. It is well known that exposure to hormonally active chemicals like DES, estradiol, or testosterone during this period can alter the sex differentiation of the brain in rodents, and we have found that the estrogenic pesticide chlordecone (Kepone) alters this developmental process like estradiol.

In addition, we have also conducted studies in which neonates were exposed later in lactation to monosodium glutamate or cadmium and lead. Although these chemicals produce rather drastic developmental alterations in our rodent models it is not immediately apparent whether these alterations correspond to human prenatal, neonatal, or pubertal stages of development.

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DISCUSSION SUMMARIES--GENERAL

HAZARDOUS WASTES AND PUBLIC HEALTH: GENERAL CONSIDERATIONS AND ANALYSIS OF EXISTING DATA SOURCES IN FLORIDA

(Robert S. Levine et al.)

DR. CROCKER: A lot of the effects you examined have a natural incidence in the population and are multifactorial in origin. If there is a chemical influence, it may be shifting a curve only 5%, 10%, or even 1%. Most teratogenic substances are discovered when a defect is noticed by a physician, and then we go back and look at the metabolism of a possible link between the chemical and the teratogen and the defect. How can one ever get around the expense of working up the metabolism of the changing background, because most of the organic chemicals (which probably include most of the carcinogens) change all the time? Industries change their solvents; they change everything. The metabolites probably number in the thousands. If you start breaking down the organic chemicals and looking at their degradation products, you end up with an enormous number of chemicals. The data aren't even available as to how to identify the peaks coming off the gas chromatograph. You have the isolation technique, and then a chemical identification technique, so it becomes an enormously expensive protocol. How does one ever get around that?

DR. LEVINE: Let me try to respond to the several questions that I heard. I think we need to investigate the multifactorial nature of these issues. Existing public health data are not specific. From a public health standpoint, we need to grasp the overall impact of residence near a hazardous waste site. That, perhaps, can be done without knowing specific chemical exposures. John Snow, after all, closed the Broad Street pump and stopped an epidemic without really knowing that germs existed, let alone vibrio cholera! As a first step, I would be interested to know whether it is really dangerous to drink tap water simply because hazardous waste has been dumped near the well field.

DR. SLOTKIN: Perhaps I'm playing devil's advocate on this, but I don't think we can avoid ultimate public policy questions about studies of this type. If we assume that birth defects are the result of very specific single events (as we usually like to think of them), and if an epidemiological study considers whole classes of compounds (99% of which may be innocuous), I can't see how the results could influence, for example, EPA policy on control of those substances or of toxic dumps. We would have to isolate the specific metabolic products that are provoking the problem. If we find a correlation and then the compounds shift from year to year, that correlation may disappear. We still don't know why it appeared or disappeared. We wind up knowing only what we know already: that contamination with anything is potentially bad!

DR. LEVINE: With respect to the question of a single, specific exposure, I would take issue with that in terms of any of the outcomes that I discussed in my presentation. The essence of the causal studies of which I'm aware is that single, specific cause-effect relationships are rare. In order to understand what's happening, one has to try and deal with a lot of factors that may or may not be producing the outcome. On a public health basis, my first priority would be a general indication of risk. Can we, for example, relate a specific adverse health outcome to the consumption of tap water?

DR. SLOTKIN: After you identify a positive risk, what is the next step? What do you do, other than to say, "We need to do something about all of these factors?"

DR. LEVINE: If one has a negative result (a likely result on a population basis), we might decide to shift our resources or priorities to an area where there is a larger impact. If we were able to identify increased risk but not the specific contaminant, we might decide to place resources into limiting the exposure: for example, by identifying the best filter to put on the water tap.

DR. CHERNOFF: A whole array of cancer epidemiological maps for states is now available (New York, Michigan, Great Lakes Registry, etc.). Invariably, such maps show that certain counties have increased incidences. The question comes down to: Where do we go from there? How do we pin it down? Is looking at these factors by county really the best way? Wouldn't it be better to begin by factoring for socioeconomic status, for exposure to agricultural chemicals, and so on? Wouldn't it be better to start an epidemiological study with a main priority hypothesis, and group the populations by that rather than by artificial boundaries?

DR. LEVINE: I agree. What I presented today are the data that are available, not the data that would be ideal. The major point I was trying to make is that we can't really make any decisions with these kinds of data. We need a more logical approach on a public health basis.

EXPERIMENTAL INTRAUTERINE FETAL GROWTH RETARDATION IN THE RAT: EFFECT OF A SINGLE DOSE OF HYDROXYUREA OR CYCLOHEXIMIDE ON THE FETUS AT TERM (Antonio J. Amortegui *et al*.)

DR. McCRORY: This is a very impressive demonstration of what an exposure might do. Do you have any data on the behavioral activities of your rats when they were 30 and 90 d old?

DR. AMORTEGUI: Unfortunately, we do not. We expect that behavior was affected. The brain was the more permanently affected organ. This may be a serious problem worldwide. It is believed that 300 million children are food-deprived and consequently may have permanent deficits in levels of physical and mental attainment.

DR. CROCKER: Obviously, your work demonstrates one method of retardation. But, if we consider the actual number of cells in the brain, was there any cell death? Was it a true inhibition?

DR. AMORTEGUI: There was clear biochemical evidence of a decrease in cell number in the brain. Histologically, we could not see this decrease. We are now doing ultrastructural morphometry, which may in part answer this question.

DR. GROSS: In noting the differences in the responses of the various organs in terms of the permanence of the reduction of DNA or protein, you alluded, I believe, to the fact that every organ has its own timetable for hypertrophy and hyperplasia. By giving it at Day 15 or Day 18 you may be catching the brain at a time when there's a very rapid increase in DNA. It may not be such a key time for the liver or heart. Before one could assume that there's some selective effect on the brain, I think, one would have to go through each organ's profile.

DR. AMORTEGUI: I agree. That study was partially done by Dr. Klionsky in England. He started the administration of these two drugs very early in pregnancy and at different dosages. Administering hydroxyurea before Day 18 or cycloheximide before Day 15 produced organ teratogenesis. Therefore, these drugs must be used in the latter part of the pregnancy. Each organ has its own pattern of growth and development. This phenomenon was demonstrated in the data I presented. We were hoping to produce a pure pattern of decreased cell size and cell number in the rat, but that was not the case.

DR. SMITH: I would like to comment on your statement that smoking or prolonged maternal corticosteroid therapy or a number of other contributory causes are themselves "causes" of growth retardation when, in fact, they shift the normal curve somewhat to the left. They do, indeed, increase the incidence of growth retardation. But, in an otherwise normal pregnancy, I suspect they would not per se cause growth retardation. The reason I bring this up is that often, in our investigation of growth-retarded infants, we find out that the mother smokes and cease all further investigation. I think the importance of these kinds of factors is that they tend to lower birth weight by 150 or 200 g.

DR. CHERNOFF: Coming back to the question of long-term effects of intrauterine growth retardation, there are a number of pertinent studies. Some of these date back to the famine in the Netherlands during World War II. In more recent studies, twins with unequally shared placentas have been followed up. Most of these studies indicate that intrauterine growth retardation is associated to some extent with intellectual impairment. It doesn't cause overt retardation, but, in the twin studies, for instance, the twin with the smaller placenta and that was smaller at birth invariably had a lower measure of intelligence by whatever test was used. So the effect does appear to be permanent.

INFLUENCE OF MATERNAL DISEASE ON THE HEALTH AND DEVELOPMENT OF THE FETUS AND NEWBORN (Euan G. Robertson)

DR. CHERNOFF: You say that you've seen a number of these arrhythmias, for example, and that they surprise you. To what extent do doctors in other hospitals look for the same effect? How much of this is possibly a real, new phenomenon, and how much is possibly just due to a serendipitous finding that has encouraged you to look for (and see) more of it? Is there any way to tell?

DR. ROBERTSON: No. I'm part of the perinatal team at a tertiary-level referral center. We found one case on our own; the other four were referred to us from other centers. We're seeing a small number out of a very, very large number of births. Are some cases missed? Very possibly.

DR. GROSS: Transient arrhythmias (particularly paroxysmal atrial tachycardia) are very common in the newborn period. As fetal monitoring becomes a more common procedure, an increasing number of these are detected antenatally. Probably they've always been there, but now we're detecting them earlier.

DR. ROBERTSON: You're probably right. But it's interesting that you don't see these very often in labor. In fact, with one patient who had a marked arrhythmia prior to labor, once labor started the arrhythmia disappeared. The baby then developed the arrhythmia again in the neonatal period. So prepartum -- not intrapartum -- monitoring is helpful here.

DR. GROSS: Maybe it disappeared in labor due to the vagal stimulation.

DR. SMITH: The infants who have RDS at term represent an interesting paradox, since RDS is supposed to be a disorder of prematurity. We all see the occasional infant with RDS, and one thing that's clear, I think, is that there's a high recurrence risk in subsequent pregnancies. That raises the question of whether it is a genetic effect, in which case we might look for other kinds of information. If it's an environmental effect, presumably it's a persisting effect in that particular mother.

DR. ROBERTSON: I question the "genetic" idea because I have a patient who has had three babies by three different fathers, and all three babies have had wet lung. Now, it may be a dominant effect. But, if it's a recessive effect (as we would expect), it shouldn't have occurred in all three pregnancies.

DR. GRABOWSKI: Experimentally in rats, we too have seen RDS without prematurity. Several hours after birth, the animals go through difficult RDS. The lungs are collapsed. But it's not associated with prematurity. Such RDS can be produced by several agents, one of which is the pesticide mirex. (My presentation focuses on the heart effects, but there are respiratory effects, too.) Dr. Daston discusses cadmium, which can do the same thing. So there are at least two environmental agents that can produce these kinds of problems. One produces hydrops fetalis; the other doesn't.

DR. BALLARD: You mentioned cases of RDS in term pregnancies of well-controlled diabetics. Might some of these be transient tachypnea with retained lung fluid?

DR. ROBERTSON: Quite possibly. Clinically, it's difficult to sort out Type 1 from Type 2 RDS. But occasionally one does see a full-blown RDS with a mature fetus. It's very uncommon, though.

DR. GARY KIMMEL: Early in your paper, you mentioned the decreased neonatal mortality. How much of that is due to our increased ability to either determine or cure disease, and how much is due to our increased ability to maintain a subnormal infant through the neonatal period?

DR. ROBERTSON: I don't think that any baby that has a structural malformation that is incompatible with life has ever been saved. The majority of neonatal practice is the support of the premature infant to the point where the organ systems all click in. There are some disease processes that the neonatologists can cure, but it's mainly support. One of the problems we face is that they now support babies down at levels that would have been totally incompatible with life 10 yr ago. This makes it difficult to determine which effects are due to outside agents, and which are due to genetic factors.

DR. GARY KIMMEL: Has there been a subsequent increase in children with developmental problems?

DR. ROBERTSON: Being a big center, we have 10,000 deliveries a year and a huge neonatal intensive care unit that follows through, under a state-mandated program, all babies born at under 1,500 g. At present, the 5-yr follow-up studies show that approximately 10% of the very small babies (<800 g) will have neurologic and other functional deficits.

DR. SMITH: But we have to look beyond our own in-house experience to the fact that the practices used in Level 3 units are spilling over into less-sophisticated levels of care. The excellent French study (and there are similar data from Canada) show an introduction of Level 3 neonatal care yielding a decrease in the number of handicaps. There's no doubt in my mind that we are saving infants to be handicapped who previously would have died, but these are more than balanced by infants who would have survived anyway but are now surviving intact.

ASSESSMENT OF THE UTILITY OF POSTNATAL TESTING FOLLOWING PRENATAL EXPOSURE TO FORTY CHEMICALS (Leon Earl Gray, Jr., et al.)

DR. SPITZER: How did you select these 42 specific agents, the dosages at which to administer them, and the end points to be examined? What was the logic behind this whole process?

DR. GRAY: The experiment was initiated with the idea of dosing through organogenesis, Days 8 to 12, and then looking at postnatal growth viability at Postnatal Days 1 and 3 as a means of prioritizing chemicals for a standard teratology bioassay. The chemicals were known teratogens or nonteratogens reported in the literature. The purpose was to determine whether viability at Postnatal Days 1 and 3 correlated well with the published reports: Would teratogens cause postnatal death while nonteratogens did not?

DR. SPITZER: Wouldn't it have been preferable to start with the known chemical structures of certain substances?

DR. GRAY: Structure-activity relationships are notoriously weak in teratology, so we started with known teratogens and nonteratogens.

DR. DASTON: Would you say that you can detect most of your effects by a couple of months, postnatally? If so, wouldn't that be more economical and informative than, say, a three-generation test?

DR. GRAY: Most of the teratogens were picked up within 3 d. Some other effects could be detected at or before weaning. Effects on the reproductive system, however, certainly can't be evaluated until after puberty; it would require 2 mo to evaluate that system. This protocol would not, however, pick up chemicals that affect spermatogenesis (as would a two- or three-generation study). Two major problems involved in this sort of experiment are control-to-control, block-to-block variability and the sheer number of comparisons that are made between treatment groups. These present difficulties with statistical interpretation that necessitate experimental replication of each subtle effect detected. A 10 or 15% change in locomotor activity on Day 22 isn't very impressive unless it's replicated. These replicate experiments, in turn, increase slightly the amount of time required to complete the protocol.

DR. McCRORY: What is the payoff, so far, in terms of defining "teratogen?"

DR. CHERNOFF: Right now, the definition is "any permanent structural and/or functional alteration induced prenatally." So it has come to mean more than strictly morphological alterations. A protocol such as Dr. Gray's picks up, in many cases, more effects than would be detected in a standard teratology test, because a standard teratology test considers morphology but not function. At least Dr. Gray's protocol includes an end point of postnatal malfunction.

DR. McCRORY: How did you select dosages?

DR. GRAY: Dosages were selected either from the available data base or from toxicity studies in which nonpregnant females were exposed to different doses, and then a dose that was minimally toxic to the dam was selected. The doses were as high as could be used without making the mother sick.

DR. KAVLOCK: The idea of this type of study is not to use the data from it to incriminate any particular compound. It's just meant to identify the potential of a compound to adversely affect the developmental process. To know what a regulatory agency should do, one would have to go on and study the chemical in more detail. Because caffeine shows up as "positive" or "negative" doesn't mean anything for caffeine per se -- what we want to know is whether the effect detected reflects what the literature says about caffeine.

DR. CHERNOFF: I'd like to follow up Dr. Spitzer's comment. This study represents the follow-up on a study to determine if a testing regime costing about $3,000 per compound could mirror results that one would expect with standard procedures costing about $65,000 per compound. The question was: If you use the dose that you know (from the literature) should produce terata, or, conversely and equally importantly, should not produce terata, will you see those effects by taking a series of shortcuts (i.e., dosing fewer days and weighing and counting animals rather than analyzing fetuses)? That was the sole purpose of this study. What Dr. Gray has shown, in this part of it, is that we missed things at Day 3 that occurred later on. We have learned, in general, a lot of interesting things about long-term follow-up.

DR. GRAY: I'm not attempting to recommend this as a protocol for EPA or for anyone else. I'm attempting to demonstrate that there are developmental effects that occur following exposure to some of these chemicals that will be missed by present procedures. There is no cohesive data base to demonstrate that postnatal testing has any value at all, and I think we're at the beginning of that.

DR. BALLARD: In view of the incidence of diaphragmatic hernia, did you weigh the diaphragms? Were they of lower weight? Did the diaphragm weight correlate with lung weight (as a possible mechanism for decreased lung growth)?

DR. GRAY: We did not weigh the diaphragms. There were many animals that had small lungs without a diaphragmatic hernia, but we didn't weigh the diaphragms.

NORMAL AND ABNORMAL STRUCTURAL DEVELOPMENT OF THE HUMAN LUNG

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INTRODUCTION

There are several maturational events that must occur before the onset of normal respiratory function in the newborn. These include structural development of the lung such that there are sufficient sites for gas exchange, maturation of the surfactant system so that sufficient expansion of air spaces is maintained, and maturation of the neuromuscular system controlling respiration. This review will consider normal structural development of the human lung and congenital malformations of the lung, especially with respect to dating the onset of the abnormality. It will also consider in some detail pulmonary hypoplasia and the factors that appear to be important in the regulation of normal structural lung growth in late gestation.

NORMAL STRUCTURAL DEVELOPMENT OF THE HUMAN LUNG

Qualitative

It is possible to date the human lung in terms of its structural maturation in much the same way that it is possible to date the kidney and brain. Structural development of the human lung extends over an exceedingly long period of time, from the beginning of the gestational period through about the first two years of life.

Lung development begins in about the fourth week of the embryonic period, when the laryngotracheal groove forms as an outpouching of the foregut (Moore 1982). A single lung bud forms at the caudal end of this groove and divides as the two primary bronchial buds form. During the fifth week these buds grow laterally into the walls of the periocardio-peritoneal canals, and the primary bronchi divide to form lobar bronchi. Segmental bronchi form in the sixth week. With this event, the proximal airways are formed, the bronchopulmonary segments are established, and the embryonic period of lung development is completed.

It is generally believed that in the next 10 weeks all the conducting airways are formed by dichotomous branching of bronchial buds, with most being formed between Week 10 and Week 14 (Bucher, Reid 1961). After this period, the airways grow only in size. During Weeks 10 through 14, the lung has a glandular appearance, with small epithelial-lined tubules disposed in abundant mesenchyma; this phase of lung growth is called the pseudoglandular phase. The respiratory epithelium begins to differentiate, with cilia appearing at about 13 weeks, and cartilage also begins to form.

The next period, Week 17 to Week 28, which approximates the midtrimester of gestation, is conventionally called the canalicular period; it is more appropriate to think of it as the period of acinar development. At this time, the basic structure of the gas-exchanging portion of the lung is formed and vascularized. Late in this period, there is enough structural development for extrauterine existence to become possible. Early in the canalicular period, the lungs have a simple internal air space configuration. The potential gas-exchanging structures are smooth-walled, blind-ending channels, lined by cuboidal epithelium and separated by relatively thick interstitial tissue of sparse cellularity. Capillaries are scattered in the interstitial tissue and they approach the epithelium in only a few places. Later, these smooth-walled channels take on an irregular internal configuration as more subdivisions of the acini are formed. At about Week 20, differentiation of the primitive epithelial-lining cells begins with the first appearance of the lamellar and multivesicular bodies associated with surfactant synthesis, identifying these cells ultrastructurally as type II cells (Lauweryns 1970). There is also beginning flattening of the epithelial cells as type I cells form. This process begins proximally in the acinus, adjacent to the last conducting airway, and over the narrow, developing air-blood interfaces.

At about Week 28, early in the period designated as the terminal sac phase, there are striking changes in the appearance of the lung, with a marked decrease in the prominence of the interstitial tissue as the saccular walls become narrower and more compact. Secondary crests appear, dividing the distal air spaces into smaller units; however, the saccular walls retain their double capillary character and the bulk of the interstitial tissue is present between two capillary networks. Cuboidal epithelial cells progressively decrease in proportion, but still remain the major component of the most distal air spaces. Alveoli may begin to appear late in this period.

In humans, there is a prenatal period of alveolar development. It begins in some lungs as early as Week 32 and is present in all by 36 weeks of gestation (Langston, Thurlbeck 1982). The process of alveolar acquisition is continuous and extends through the neonatal period and early childhood.

Quantitative

Quantitative data for normal human lung growth are now available. These data permit somewhat more precise statements about structural development. The number of alveoli in the human lung at term is about 55 million, but there is considerable variability in the normal data; the range is from 10 to 150 million (Langston, Thurlbeck, unpublished observation). This wide range persists through postnatal lung growth, but it appears that most if not all alveoli form early in childhood (Fig. 1) and that alveolar acquisition is complete by about age two (Thurlbeck 1982).

Other quantitative changes also occur during late gestation before the appearance of alveoli. These include a rapid fall in the thickness of the air space wall (Fig. 2), with a decrease in wall thickness from about 45 um at 20 weeks to about 17 um at 32 weeks, before the onset of alveolar development (Langston, Thurlbeck 1982). There is relatively little change through the rest of gestation. Another major change is that occurring in the internal

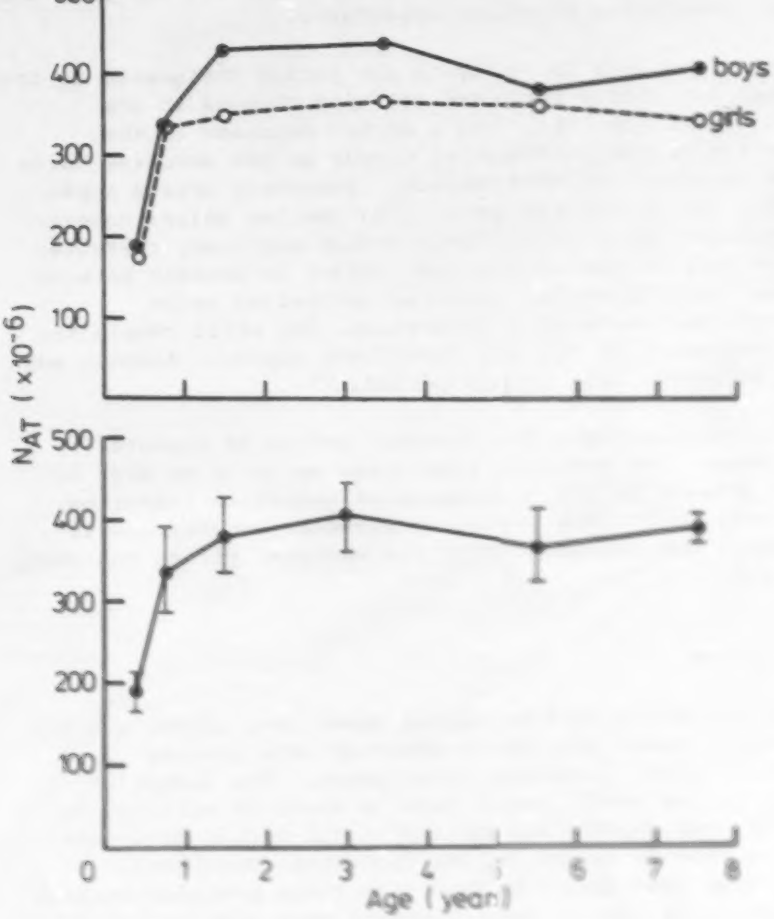

Fig. 1. The mean value of the total number of alveoli (N_{AT}) is plotted against age for children in the bottom section; in the upper section, boys and girls are plotted separately. No significant difference exists in children between ages 1 to 2 and 7 to 9 years. The total number of alveoli is larger in boys than in girls after age 1; this is entirely due to larger lung volumes in boys. [Reproduced with permission from: Thurlbeck 1982. Copyright 1982 by Thorax. A. Brewis and H. Matthews, eds.]

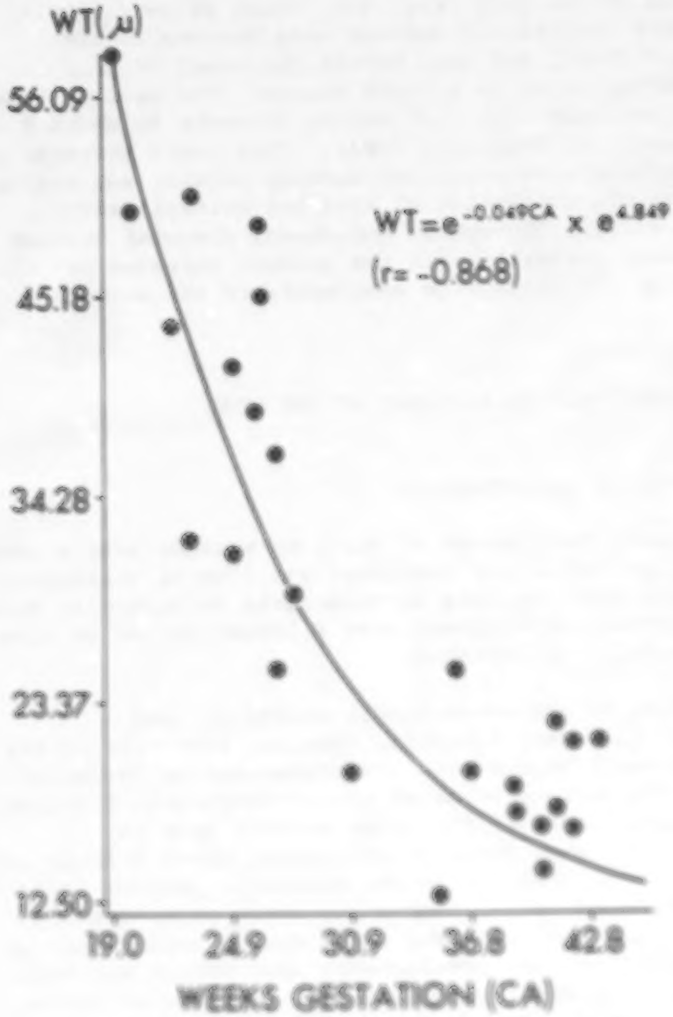

Fig. 2. The mean wall thickness (WT) of fetal human lungs plotted against gestational age. The best-fitting curve is drawn, and its equation and the correlation coefficient (r) are indicated. The wall thickness drops rapidly prior to alveolar development and then remains stable. [Reprinted with permission from: Langston, Thurlbeck 1982. Copyright 1982 by Masson Publishing USA, Inc., New York.]

surface area of the lung (Fig. 3). There is very little change in the respiratory surface area between 20 and 28 weeks gestation, and then before the onset of alveolarization there is a rapid change: the surface area increases from less than 1 m^2 before 28 weeks to about 4 m^2 at term (Langston, Thurlbeck 1982). This rapid increase in alveolar surface area continues through infancy and begins to slow with the completion of alveolar multiplication (Thurlbeck 1982). Throughout childhood, alveolar surface area increases gradually with the gradual increase in alveolar size and perhaps in complexity of the alveolar wall.

ABNORMAL STRUCTURAL DEVELOPMENT OF THE LUNG

Dating Pulmonary Malformations

Structural development of the lung extends over a long period of time and is not completed until early childhood. This suggests that the lung is vulnerable to injury in terms of its structural development over a longer period of time than is generally appreciated.

According to the conventional scheme of lung development, pulmonary agenesis, abnormal formation of the tracheoesophageal septum with the production of laryngeal clefts and the various forms of tracheoesophageal fistulae, and the abnormalities of the major bronchi seen in association with some forms of congenital heart disease, all must occur early, that is, in the embryonic period. This is also the period of organogenesis for other major organ systems, and if renal agenesis has a direct rather than an indirect effect on lung development, that effect may begin in this period as well. When problems arise later during the pseudoglandular period, the period of bronchial development, bronchial number should be affected. This effect is seen with the diaphragmatic abnormalities whose effects are initially exerted in this period. Other abnormalities of bronchial formation, including most congenital pulmonary cysts, also should have their origin during this phase. In the canalicular period, the pulmonary acini, the gas-exchanging components of the lung, are formed. Problems at this time lead to the deficiencies in acinar growth which we recognize as pulmonary hypoplasia:

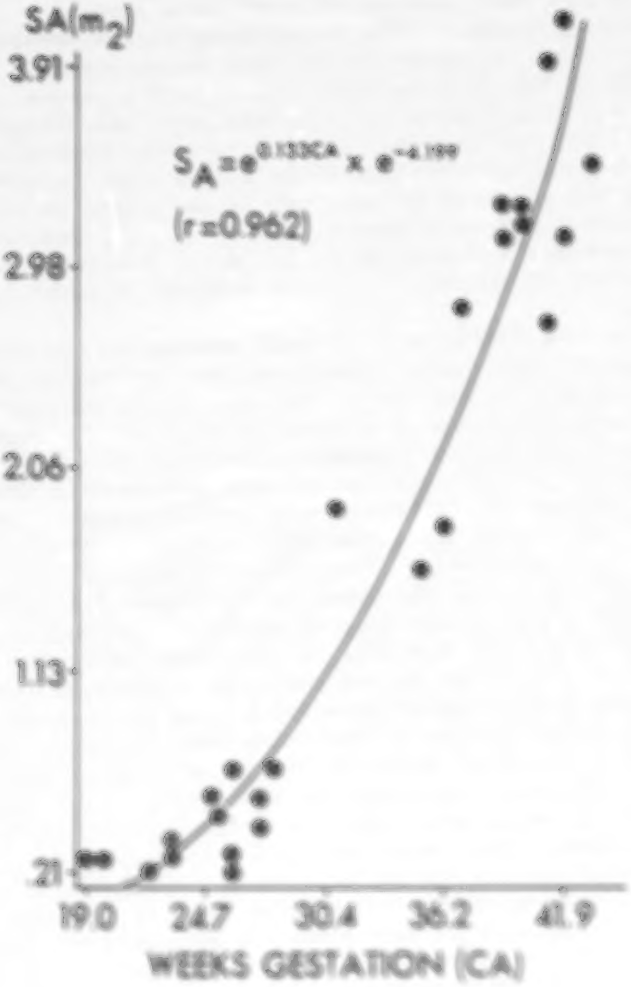

Fig. 3. The internal surface area (SA) of human fetal lungs plotted against gestational age. The best curve has been drawn, and its equation and the correlation coefficient (r) are indicated. The surface area shows little increase before 28 weeks gestation and then shows a rapid rise through the last trimester. [Reprinted with permission from: Langston, Thurlbeck 1982. Copyright 1982 by Masson Publishing USA, Inc., New York.]

the lung is too small and it does not have enough gas-exchanging tissue. Late in the canalicular period and through the rest of gestation not only is structural development of the lung at risk, but injuries to the lung at this time may interfere with the functional maturation of the respiratory epithelium. Because alveolar multiplication continues throughout infancy, it is likely that injuries during infancy may also be manifest as deficiencies in alveolar number and development, and these deficiencies may have consequences in terms of an increased incidence of and susceptibility to chronic pulmonary disease in later life.

Much of the conventional scheme depends on the truth of the fundamental hypothesis underlying our conception of structural growth of the lung. The hypothesis is that growth is accomplished at the terminal ends of the branching structures of the lung. Proximal structures will have completed their branching morphogenesis before more distal structures arise. There is now some evidence that this may not be so. This evidence comes from studies on the epithelial differentiation of the developing mouse lung (Ten Have-Opbroek 1981). In the mouse, a lung-specific antigen has been identified in the type II alveolar epithelial cell. The antigen is present not only in the adult mouse but also in the fetus. It occurs in the cuboidal epithelium that lines the developing acinar structures, and it is never present in the epithelium of the conducting airways. It appears in the terminal portions of the developing mouse lung before bronchial branching is completed. This suggests that the acinar structures begin to develop before bronchial branching ceases. This would mean that at least some bronchial branches are formed by lateral budding from already developed airways, rather than only by terminal budding. If this work is confirmed, we may have to alter our ideas about how the lung grows and the conclusions about when in development structural abnormalities can occur.

Pulmonary Hypoplasia

The major structural anomalies of the lung are rare and few in number, and there are few data concerning their incidence. Most occur early in the embryonic period. The exception is seen in situations that result in pulmonary hypoplasia. The studies of pulmonary hypoplasia have led to the current understanding of the controls of late gestational structural development in the lung. Pulmonary hypoplasia occurs in a variety of settings, usually as a component of malformation syndromes manifest as secondary involvement of the lung through compromise of its growth in late gestation. The incidence of pulmonary hypoplasia, reported in terms of percentage of neonatal and perinatal autopsies, has been seen in 10 to 15% of such autopsies (Page, Stocker 1982; Wigglesworth, Desai 1982). One recent study shows an increase in its incidence from 12 to 25% (Moessinger _et_ _al_. 1983).

The tendency for pulmonary hypoplasia to occur as a secondary condition associated with other abnormalities has suggested that there are common factors in these situations that affect lung growth. The factors that have been investigated in terms of the controls of late gestational lung growth include available space, amniotic and lung fluid volume, and the role of fetal breathing movements.

Available space (fetal compression). Pulmonary hypoplasia is known to be associated with space-occupying lesions of the fetal thorax. Although many intrathoracic lesions may limit lung growth, the most common of these is congenital diaphragmatic hernia, with an incidence of about 1 in 2200 births (Rubin 1969). In various series, lung maturation in congenital diaphragmatic hernia shows a spectrum from normal (Moore _et_ _al_. 1957; McNamara _et_ _al_. 1968) to severe hypoplasia (Areechon, Reid 1963; Kitagawa _et_ _al_. 1971; Boyden 1972; Hislop, Reid 1976; Thurlbeck _et_ _al_. 1979; Cooney, Thurlbeck 1983). The hypoplasia is generally thought to be due to deficiencies in bronchiolar generation (Kitagawa _et_ _al_. 1971), but in some series acinar hypoplasia has also been a significant component (Reale, Esterly 1973; Askenazi, Perlman 1979; Cooney, Thurlbeck 1983). The favored theory for the genesis of pulmonary hypoplasia in congenital diaphragmatic hernia is that the developing lung is compressed directly by the herniated abdominal viscera, beginning early in gestation before bronchial branching is complete (Boyden 1972; Reale, Esterly 1973). Pulmonary hypoplasia has been created in fetal lambs following the surgical creation of diaphragmatic hernia (Kent _et_ _al_. 1972), and by the simulation of such an event by inflation of an intrathoracic balloon (Harrison _et_ _al_. 1980). This suggests that direct compression is causally related to the hypoplasia seen in congenital diaphragmatic hernia.

There are, of course, situations that do not fit this model. The most striking of these seem to be cases of unilateral or bilateral agenesis of the diaphragm, in which abdominal viscera also fill one (or both) hemithorax(es). These would seem to be instances of compression as severe as any seen with congenital diaphragmatic hernia, yet clinical reports have shown long-term survival after repair of these defects in some cases (Neville, Clowes 1954; Benjamin 1963; Eichelberger et al. 1980; Geisler et al. 1977). And one case of bilateral diaphragmatic agenesis which was assessed quantitatively showed no evidence of pulmonary hypoplasia (Toran, Emery 1981). Thus, although compression seems to be an important factor in the genesis of pulmonary hypoplasia, it apparently does not invariably cause limitation of lung growth.

Amniotic and lung fluid volume (oligohydramnios). About ten years ago it became generally recognized that the nonrenal features of Potter's syndrome (the characteristic facial appearance, positional malformations of the hands and feet, late fetal growth retardation, and pulmonary hypoplasia) were manifestations of the oligohydramnios that regularly accompanies renal agenesis (Thomas, Smith 1974), and not a consequence of multiple early mesodermal defects. Pulmonary hypoplasia and the other nonrenal features of Potter's syndrome regularly occur in oligohydramnios, due not only to deficient fetal urine production, but also to chronic leakage of amniotic fluid (Bain et al. 1964; Perlman et al. 1976). Models of oligohydramnios produced by chronic drainage of amniotic fluid also show these same features (Symchych, Winchester 1978). They are not present in cases of renal outflow obstruction or renal agenesis in which deficient amniotic fluid is not a factor (one affected and one unaffected mono-amniotic twin (Marras et al. 1983)), and when another malformation such as congenital cystic adenomatoid malformation (Krous et al. 1980) or esophageal atresia (Thomas, Smith 1974) co-exists with renal agenesis. The nonrenal features of Potter's syndrome, including the pulmonary hypoplasia, have been interpreted as being caused by compression of the fetus by the uterine wall in the absence of amniotic fluid (Thomas, Smith 1974); the other possible cause that is generally considered is that of an altered pressure gradient with enhanced outflow of lung liquid. The lung pathology of only two human cases of chronic leakage of amniotic fluid has been critically examined. In both cases, there was no structural development beyond the time of onset of fluid leakage (Perlman _et_ _al_. 1976).

Experiments involving tracheal ligation and chronic tracheal drainage in fetal sheep have stressed the importance of lung fluid in late gestational pulmonary growth (Alcorn _et_ _al_. 1977). Tracheal ligation produces enlarged lungs, with both increased fluid and increased tissue. Type II cells, the probable source of fetal lung liquid as well as surfactant, were less numerous. Chronic tracheal drainage produced the opposite effects: small lungs with thicker alveolar walls and fewer alveoli but with type II cells present in increased numbers.

Both clinical and experimental results suggest that lung fluid and/or amniotic fluid play an important role in the growth of the lung _in_ _utero_. This suggestion has implications for current and developing obstetric practices. The British Working Party on Amniocentesis has demonstrated two unanticipated hazards of midtrimester amniocentesis: an increased incidence of unexplained respiratory difficulties (rising from 0.4% to 1.3%) and an increased incidence of major postural deformities of the musculoskeletal system (from 0.2% in controls to 1%) (Medical Research Council Working Party on Amniocentesis 1978). It is likely that both of these effects are related to changes in amniotic fluid volume following the procedure. A preliminary report of a study of the effects of amniocentesis in monkeys (Hislop, Fairweather 1982) showed that early amniocentesis (corresponding to Weeks 14 to 17 of human gestation) produced significant reduction in both birth weight and lung volume. These reductions were not seen with late amniocentesis (corresponding to 22 to 25 weeks gestation); however, in both groups there was a significant reduction in alveolar number and an increase in alveolar size.

The lungs of infants with renal agenesis and dysplasia show morphologic abnormalities beyond a simple reduction in volume (Hislop _et_ _al_. 1979). With oligohydramnios and renal abnormalities there is a reduction in total lung volume, airway generation, and alveolar size, with a less striking reduction in alveolar number. Amniotic fluid deficiency due to lack of urine production by the fetus is not likely a factor at the stage in gestation when airway formation takes place; some other mechanism must be considered. Because the lung and the kidney both begin their development at the same time, it has been suggested that they may both be affected by a common insult, there may be a congenital mesodermal defect, or the kidney may play a role in lung development more directly than through its effects on amniotic fluid volume. A possible mechanism by which the kidney may affect lung development early is through proline and collagen production. During fetal development the kidney is an important source of proline, which is produced by a renal arginase. Proline is necessary for collagen synthesis. Proline analogues inhibit collagen synthesis and result in decreased bronchial budding (Alescio 1973). Thus, insufficient collagen due to decreased available proline may be a factor in the pulmonary hypoplasia associated with some renal abnormalities. It has been shown experimentally that the injection of nephrotoxins into chick embryos results in decreased proline and collagen production and in lung hypoplasia (Clemmons 1977).

The supply of nutrients and structural components may be important in lung growth. Maternal malnutrition has been suggested as a cause of delayed fetal lung maturation. Most of the evidence for this suggestion has come from animal experiments, and the role of nutritional status in the human is not well understood. Offspring of malnourished rats have shown morphological changes in their lungs at birth (Faridy 1975; Curle, Adamson 1978), including hypocellularity, increase in air space size, delay in epithelial differentiation, decreased surfactant content, and delayed alveolar differentiation. Postnatal undernutrition in rat pups causes decreases in lung weight, body weight, lung protein, and RNA and DNA, but does not apparently affect the morphological structure of the lung (Frank, Groseclose 1982).

Fetal respiratory movements. At about the same time that oligohydramnios and fetal compression were being suggested as factors in many cases of pulmonary hypoplasia, a separate series of investigations led to the recognition of the importance of fetal breathing movements for lung growth. The neuromuscular activity that occurs when respiration is established in the newborn is not a completely new activity. The fetus shows breathing movements beginning at about the end of the first trimester (Boddy, Dawes 1975). They appear to be related to REM activity in the fetus (Dawes et al. 1972), and their frequency is depressed by a variety of factors, including hypoxia, drugs, maternal smoking, and stress (Boddy, Dawes 1975; Manning, Feyerabend 1976; Fox et al. 1978). Because pulmonary hypoplasia is seen in situations in which neuromuscular activity is compromised, it seemed promising to investigate the role of fetal breathing movements in lung growth. This has been done two ways: 1) The effects of phrenectomy have been studied in fetal sheep (Alcorn et al. 1980) and fetal rabbits (Somasundaram, Prathap 1975), and 2) the effects of cervical cord injury have also been studied in fetal rabbits (Wigglesworth, Desai 1979).

Unilateral phrenectomy in fetal rabbits creates diaphragmatic muscular atrophy, but it does not appear to affect pulmonary development (Somasundaram, Prathap 1975). However, bilateral phrenectomy in fetal sheep produces profound changes in lung growth, with cessation of structural development following phrenectomy, although epithelial cell differentiation continues apparently normally (Alcorn et al. 1980). Growth is impaired but epithelial maturation is normal. The effects of cervical cord section in fetal rabbits, when the phrenic nerve nucleus is below the level of the section (Wigglesworth, Desai 1979), are similar to the effects of bilateral phrenectomy. With cervical cord section, the phrenic nerve remains intact, the diaphragm does not atrophy, and normal diaphragmatic muscle tone is maintained. That this was not due to a change in lung-liquid secretion related to the absence of fetal breathing movements was studied by concomitant cord section and tracheal ligation. In this situation the lungs enlarge and show essentially no difference from normal controls. Both these studies and those related to oligohydramnios suggest that distention of the fetal lung with liquid is important for structural growth.

The way in which fetal breathing movements affect lung growth may be through transiently increasing the total volume of the lung. Stretch has been proposed as an important mechanism in the initiation of adaptive lung growth postnatally, and it seems reasonable to suggest that similar mechanisms may operate in late gestation. In fetal sheep lung inflation abolishes and lung deflation increases spontaneous respiratory movements (Ponte, Purves 1973). Also, the volume of fluid recovered from the lung after fetal breathing movements is consistently greater than that recoverable without preceding fetal breathing (Murai et al. 1983).

Although it may seem initially that lack of fetal breathing movements and oligohydramnios produce their effects through a similar mechanism (failure to distend potential air spaces sufficiently to stimulate lung growth), the morphologic effects of these two situations are said to be different (Wigglesworth, Desai 1982). It has been suggested that with oligohydramnios resulting from any cause, the lungs show narrow airways, impaired maturation of interstitial tissue, and failure of normal elastic tissue development, as well as decreased lung lecithin and palmitate. Alternatively, when amniotic fluid volume is normal or increased in association with pulmonary hypoplasia, the lungs, although small, are of appropriate maturity for gestational age. This suggests that different mechanisms are involved in the limitation of lung growth in these two situations. Structural abnormalities in pulmonary hypoplasia are variable and do not appear to correlate with the degree of hypoplasia. It does seem that infants with pulmonary hypoplasia without renal abnormalities, and possibly all those without oligohydramnios, have structurally more normal, although small, lungs. These infants may have a better potential for more normal postnatal development.

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THE REGULATION OF FETAL LUNG DEVELOPMENT

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INTRODUCTION

Respiratory Distress Syndrome (RDS) of the newborn is believed to be due to immaturity of the fetal lung, particularly the pulmonary surfactant system. The significant mortality and morbidity caused by this disorder has focussed attention on those factors which influence lung maturation and on attempts to prevent RDS by pharmacologic acceleration of fetal lung development. Although a considerable amount of information relating to the hormonal regulation of fetal lung maturation has been obtained during the last 10 years, this process is still not completely understood. The evidence suggests that the final stages of fetal lung development, including the maturation of the pulmonary surfactant system, are under hormonal control (Gross 1979). A number of hormones have been shown to be involved. They include corticosteroids, thyroid hormone, cyclic AMP, and beta adrenergic agonists (Table 1). The roles of other hormones such as estrogen and prolactin are more controversial. In addition, it is possible that insulin delays lung maturation so that the final rate of lung development may depend on a complex interaction between these and possibly other hormones. Little is currently known of the mechanism of action of these hormones. It has not yet been established whether each hormone acts on one enzyme specifically or whether the activities of a number of enzymes are influenced simultaneously. In addition, although it is clear that hormones influence the rate of

Table 1. Hormonal Regulators of Surfactant Synthesis and Secretion

Stimulators	Inhibitors	? Physiologically Significant Agents
Corticosteroids	Insulin-hyperglycemia	Estrogen
Thyroid hormones		Prolactin
Beta-adrenergic agonists		
Methylxanthines and cyclic AMP		
Epidermal growth factor		

lung development, it has not been shown that they initiate it. In this review the initiation of fetal lung maturation and the later modulation of this process by hormones will be discussed.

INITIATION OF FETAL LUNG MATURATION

The levels of a number of circulating hormones increase during the last trimester of gestation. In the fetal rat, corticosteroids, thyroid hormones, and prolactin increase significantly after 18 days of gestation. There has been some debate in the literature as to whether the rise in the level of circulating hormones precedes the developmental increase in surfactant production (Ballard et al. 1978a; Hauth et al. 1978; Kitterman et al. 1981). However, the reported temporal associations between rising hormone levels and some parameters of pulmonary surfactant production do not necessarily imply that there is a causal relationship between these phenomena.

We undertook a study to determine whether lung maturation might be initiated by factors within the lung itself (Gross, Wilson 1983). The biochemical and morphological development of fetal rat lung was examined in a lung explant system with particular emphasis on the synthesis of the surfactant phospholipids. The lungs were cultured in a constant unchanging environment in which no or minimal amounts of hormones were present, and we were therefore able to observe their maturation in a system where there could be no surge in the levels of hormones or other factors. The chemically defined culture medium (Waymouth's MB 752/1) contained either 0 or 1% fetal bovine serum. Growth and morphologic maturation of explants of 13- through 20-day fetal rat lung continued in the explant system. (Term in the rat is 22 days.) There is normally a surge in the rate of incorporation of precursors into disaturated phosphatidylcholine (DSPC), the major surface-active component of pulmonary surfactant, after 20 days of gestation in the rat. We found that irrespective of the gestational age of the fetal lungs used in the culture system, there was a similar surge in DSPC synthesis at an equivalent gestational age of 20 days (e.g., 13-day lung after 7 days in culture or 16-day lung after 4 days). The increase in the rate of precursor incorporation was associated with an increase in DSPC accumulation in the lungs. This suggests that the timing of the initiation of DSPC synthesis is dependent on the chronological age of the lung tissue and not on the duration of the culture period. However, the magnitude of the increase in DSPC synthesis in cultures of earlier gestation (13- through 16-day) lungs was less than that which was seen *in vivo* or in explants of late gestation lungs (18 through 20 days). The late gestation lungs and those examined *in vivo* may have shown greater increases in DSPC synthesis because they had been exposed to circulating hormones, whereas the early gestation lungs had not. This explanation would suggest that although hormones are not necessary for the initiation of DSPC synthesis, they do enhance this process, and for the full expression of DSPC synthesis hormones must be present. Another parameter of fetal rat lung maturation that increases *in vivo* is glucocorticoid binding. We found that specific nuclear dexamethasone binding increased in explants of 16-day lung at approximately the same rate as *in vivo*, suggesting that a surge in the level of hormones was not necessary for this process.

Since it appeared that the signal for the initiation of lung maturation was located in the lung tissue itself, the next step was to determine in which cells it was located. Earlier studies (Smith 1979) had suggested that corticosteroids enhance DSPC synthesis by stimulating mesenchymal cells to produce a factor, the fibroblast pneumonocyte factor, which then stimulated the alveolar type II cells to produce surfactant. To determine whether a similar paracrine mechanism could be involved in initiating lung maturation we removed 50% or 75% of the mesenchymal tissue from explants of 14-day lung. Even after removal of most of the mesenchymal tissue, there was an increase in the rate of choline incorporation into DSPC in the cultures, suggesting that the message for the initiation of lung maturation is most likely contained within the epithelial cells. The signal may be preprogrammed genetically and located in the DNA of these cells.

We therefore concluded that the stimulus for the initiation of fetal lung maturation resides in the lung tissue itself. The role of circulating hormones is probably later modulation of this process.

HORMONAL REGULATION OF FETAL LUNG DEVELOPMENT

Comparison of Effects of Glucocorticoids and Thyroid Hormone

Although glucocorticoids and thyroid hormones have been shown to enhance surfactant production in fetal animals, the precise mechanism of action of these hormones has not yet been resolved. Specific receptors for glucocorticoids (Ballard *et al*. 1978b) and thyroid hormone (Lindenberg *et al*. 1978) have been described in the fetal lung, and it seems likely that they act by combining with these receptors. It has not been established, however, whether there is only one specific rate-limiting step in lung phospholipid synthesis that is stimulated by all the agents that enhance lung maturation, or if these agents act at many sites. As a first step in investigating whether these agents acted through common or different mechanisms, we examined their influence on the pattern of precursor incorporation into a number of phospholipids in fetal rat lung organ cultures (Gross *et al*. 1980). It was assumed that similarities in the effects of these hormones would suggest a common mode of action, whereas differences might indicate action by different mechanisms. We found that although dexamethasone and thyroid hormones stimulated the rate of choline incorporation into phosphatidylcholine (PC), dexamethasone treatment resulted in a greater increase in incorporation into the disaturated than into the unsaturated species, whereas thyroid treatment had the opposite effect. When the distribution of acetate in the various phospholipid fractions was examined we found that dexamethasone increased the percentage of radioactivity in the surfactant-associated phospholipids, DSPC, and phosphatidylglycerol; whereas thyroxine did not produce this effect.

The one area in which a common effect was observed was glycogen content. Exposure to dexamethasone or thyroxine resulted in a decrease in lung glycogen (as was the case with the other agents which have been reported to accelerate lung maturation, aminophylline and estrogen). Conversely, insulin, which may be an inhibitor of surfactant production (Gross et al. 1980), increased glycogen content in the lung organ cultures. These findings support the hypothesis that glycogen provides substrate for phospholipid synthesis in the developing lung.

These data indicated that although glucocorticoids and thyroid hormone acted directly on the fetal lung, there were differences in the pattern of incorporation of substrates into DSPC and phosphatidylglycerol. This suggested that these agents were acting at different biochemical sites.

Glucocorticoid-Thyroid Interactions

There is considerable evidence that glucocorticoids interact with other hormones in a number of organs. Triiodothyronine (T_3) - glucocorticoid interactions have been described in several systems including the well documented enhancement by T_3 of glucocorticoid-induced production of growth hormone in rat pituitary cell cultures (Samuels et al. 1977). Since glucocorticoids and T_3 appeared to be acting at different biochemical sites in the fetal lung, it was possible that additive or supra-additive interactions between these two agents were possible. We initially attempted to establish whether these interactions occurred in culture (Gross, Wilson 1982). This information was important not only from the biological but also from the clinical perspective. Recent clinical studies of dexamethasone administration for preventing RDS of the newborn have suggested that glucocorticoids might not be as effective for this purpose as was previously believed (Collaborative Group on Antenatal Steroid Therapy 1981). Now that favorable developmental follow-up studies of infants treated antenatally with glucocorticoids have been published, concerns regarding the use of these hormones to accelerate fetal lung maturation have turned from the issue of toxicity to that of effectiveness (MacArthur et al. 1982). There is considerable interest in establishing whether combined hormone therapy might be more effective than administration of glucocorticoids alone. We examined the interactions between T_3 and dexamethasone in explants of fetal rat lung. Combination of 100 nM dexamethasone with 100 nM T_3 (the concentrations which produced maximal effects) resulted in additive interactions. T_3 treatment alone resulted in 37% stimulation of the rate of choline incorporation into PC, dexamethasone alone in 100% stimulation, and the two agents together in 197% stimulation. The combined effect was significantly different from that of either hormone alone. We also found that both hormones significantly stimulated the activity of cholinephosphate cytidylyltransferase (Gross et al. 1983), an enzyme that appears to be the rate-limiting step in PC synthesis in the lung. However, the combined hormone effect, which was equal to the sum of the individual hormone effects, was not significantly different from that produced by dexamethasone alone.

Because of the possibility of using combined hormone treatment to accelerate lung maturation in humans, we also examined glucocorticoid - T_3 interactions in fetal rat lung in vivo (Gross et al. 1983). Since thyroid hormones do not cross the placenta to any great extent in most species, most studies of thyroxine administration to fetal animals have involved direct injection of the fetuses. However, it is known that direct fetal injection is a stress which in and of itself can produce accelerated lung maturation. We injected the pregnant rats with T_3 and found that there was significant passage of T_3 across the placenta to the fetuses. Pregnant rats were injected with T_3, betamethasone, or a combination of these agents on gestational Days 18 and 19, and the fetuses were studied on Day 20. Injection with the optimal doses of T_3 or betamethasone resulted in about 35% stimulation of choline incorporation into PC when either agent was given alone. Administration of both hormones together resulted in a 70% increase, an additive effect. T_3 injection did not produce elevated fetal serum corticosterone levels as had been the case when the thyroid analogue DIMIT, which readily crosses the placenta from mother to fetus, had been used in fetal rabbit studies (Ballard et al. 1980).

The finding of an additive interaction between T_3 and dexamethasone in vivo is consistent with the data of Hitchcock (1979), who found that metyrapone treatment or adrenalectomy decreases the effect of T_4, injected directly into the fetuses, on the morphologic maturation of fetal rat lung. Dow and Torday (1982) have also reported that, whereas direct injection of T_4 into fetal rats produced no greater stimulation of choline incorporation into DSPC than did saline, T_4 injection did enhance the effect of directly injected dexamethasone. These studies were, however, complicated by the use of direct hormone injections.

To explore possible mechanisms underlying these observations, we examined the glucocorticoid - T_3 interaction at the level of the cytoplasmic glucocorticoid receptor, nuclear translocation of the receptor steroid complex and at the translational level. The enhancement of glucocorticoid action by T_3 could not be accounted for by any change in cytoplasmic or nuclear binding of this steroid. T_3 treatment in vitro or in vivo had no effect on glucocorticoid binding. (This finding is similar to that which has been reported previously in pituitary cells. In that system T_3 did not produce any change in glucocorticoid binding or vice versa.) There were, however, interactions at the enzyme level. Fetal lung cholinephosphate cytidylyltransferase activity was stimulated by both T_3 and glucocorticoids in vitro and in vivo. Administering both hormones together resulted in an increase in enzyme activity which was equal to the sum of that produced by the individual hormones. Fetal lung cholinephosphate cytidylyltransferase activity is increased by inclusion of acidic phospholipids in the reaction mixture. The stimulatory effects of these hormones above control values were abolished if phosphatidylglycerol, an acidic phospholipid, was added to the incubation mixture. This suggests that the hormones produced their effect by activating the enzyme rather than by increasing its amount. The physiological significance of these hormonal effects on enzyme activity is still uncertain.

The work of Smith and Sabry (1982) suggests that T_3 increases the sensitivity of the alveolar type II cells to the fibroblast pneumonocyte factor, which is released by mesenchymal cells in response to glucocorticoid stimulation. This provides another mechanism whereby these hormones may interact to produce their effects.

Our data therefore suggest that there is an additive interaction between T_3 and corticosteroids with regard to some parameters of lung phospholipid synthesis. They support the view that combined hormone administration may be more effective than single hormones in preventing respiratory distress syndrome in humans. However, we feel that more information is required before clinical use of combined hormone therapy should be considered.

CONCLUSIONS

A number of hormones act on the fetal lung and accelerate its maturation. However, it appears that hormones may not be involved in the initiation of lung development but rather in later modulation of this process. Corticosteroids have been used with some success to accelerate lung maturation and thereby prevent respiratory distress syndrome in humans. It is possible that administration of combinations of hormones, for example, glucocorticoids and thyroid hormone, may prove more effective than steroids alone in preventing respiratory distress syndrome. If further animal studies continue to be encouraging, clinical trials of combined hormone administration should be feasible in the future.

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HORMONES AND RECEPTORS IN DEVELOPING LUNG

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INTRODUCTION

This presentation reviews effects of various hormones on the development of specific respiratory functions in the fetal and neonatal lung. Current information regarding the receptor systems for glucocorticoids, thyroid hormones, and β-adrenergic agonists will be summarized. Discussion of abnormal lung development mediated by hormonal status will be primarily speculative because information on this topic is limited.

RESPIRATORY FUNCTIONS UNDER HORMONAL REGULATION

Morphological development of the lung proceeds through glandular, canalicular, and alveolar stages (Gross 1983; Langston 1983). Several hormones cause precocious maturation of lung structure, particularly during the latter two stages (Ballard 1980; Ballard 1982). Although lungs will mature structurally in the absence of hormone *in vitro* and probably also *in vivo*, hormonal deficiency during fetal life results in a less mature lung at all stages of terminal differentiation.

The pulmonary surfactant system is also under multi-hormonal control. Surfactant is produced by the rough endoplasmic reticulum and Golgi apparatus in type II epithelial cells. This complex mixture of lipids and specific proteins is characterized by a high disaturated phosphatidylcholine content and the presence of phosphatidylglycerol. Newly synthesized surfactant is stored in intracellular lamellar bodies that fuse with the cell membrane for release of the packaged surfactant. In the alveolus, surfactant initially assumes a multi-tubular arrangement termed tubular myelin. A monolayer of phospholipid that coats the aqueous interface of the alveolus is subsequently formed, reducing surface tension and preventing alveolar collapse on expiration (Goerke 1974; King 1982). The precise composition of the monolayer is unknown, but current studies suggest that it may contain only selected components of the surfactant complex. The life cycle of surfactant appears to include recycling of material back into type II cells for reutilization. Both the synthesis and release of surfactant are influenced by hormones (Ballard 1980; Ballard 1982).

The fetal lung produces and secretes a liquid that has a higher concentration of chloride and lower levels of calcium, magnesium, bicarbonate, and protein than plasma. The composition of fluid is constant through the third trimester, except for an increase in potassium in the last two weeks (Table 1). Lung fluid is transported into the potential airspaces through the action of a chloride pump (Olver, Strang 1974). Clearance of lung fluid is necessary for normal air breathing after birth. This process normally begins with the onset of labor and involves both decreased fluid production and increased reabsorption from the potential airspaces (Walters, Olver 1978; Bland et al. 1979; Kitterman et al. 1979). Uptake of fluid apparently occurs, at least in part, via a sodium pump in type II cells (Mason et al. 1982). Both fluid production and reabsorption are influenced by β-adrenergic compounds (Walters, Olver 1978).

While it has been known for some time that the fetus actively makes breathing movements during later gestation, the relationship between breathing movements and lung growth has been explored only recently. Experimentally, fetal breathing is decreased by phrenectomy, thoracoplasty (to decrease the amplitude of breathing excursions), and spinal cord section. With each of these maneuvers, the rate of lung growth decreases substantially (Table 2). These observations indicate that fetal breathing movements influence the rate of lung growth and suggest that decreased breathing movements can produce lung hypoplasia. Several

Table 1. Electrolyte Composition of Lung Fluid and Plasma in Fetal Sheep

	Plasma	Lung Fluid
Sodium (meq/l)	146.1 ± 0.5	147.8 ± 1.1
Chloride (meq/l)	110.1 ± 1.3	153.1 ± 1.1
Total calcium (mg/dl)	12.1 ± 0.4	2.0 ± 0.1
Magnesium (mg/dl)	2.3 ± 0.1	<0.5
Bicarbonate (meq/l)	27.0 ± 0.7	2.1 ± 0.3
Total protein (mg/dl)	4.5 ± 0.2	<0.06
pH	7.39 ± 0.1	6.23 ± 0.02
Potassium (meq/l)	3.65 - 5.06	4.10 - 5.06[a] 5.06 - 8.96[b]

[a] For 120 to 135 days gestation.

[b] Progressive increase between 135 and 148 days gestation. Data are for samples (n = 6 to 129) collected between 120 and 148 days gestation except for lung fluid potassium. Mean ± SE values are shown except for plasma potassium (range). Data are from Mescher *et al*. 1975.

hormones, especially the prostaglandins, appear to influence fetal breathing movements.

Several non-respiratory hormonal functions of the lung, including inactivation of circulating hormones (e.g., monamines, bradykinin, certain prostaglandins) and generation of active hormones (e.g., conversion of angiotensin I→II, release of prostacyclin, and conversion of inactive cortisone to physiologically active cortisol) will not be discussed in this paper.

GLUCOCORTICOIDS

Administration of glucocorticoids to the fetus accelerates lung maturation, and hypophysectomy retards development (Ballard 1980; Ballard 1982). Findings to date are consistent with exogenous glucocorticoids influencing the rate of maturation, not the pattern of differentiation. This interpretation is supported by considerable evidence

Table 2. Fetal Breathing Movements and Lung Growth

Procedure	Fetal Breathing Movements	Wet Lung Weight (% body weight)	Reference
Sheep			
Phrenectomy	Absent	1.9 ± 0.2	Fewell *et al.* 1981
Sham	Normal	2.4 ± 0.3	
Cord section C$_{2-3}$	Absent	1.6 ± 0.2	Liggins *et al.* 1981
Cord section C$_{7-8}$	Normal	2.6 ± 0.8	
Control	Normal	2.4 ± 0.4	
Thoracoplasty	↓Amplitude	1.5 ± 0.2	Liggins *et al.* 1981
Control	Normal	2.3 ± 0.1	
Rabbit			
Cord section C$_{1-3}$	Absent	1.4 ± 0.1	Wigglesworth, Desai 1979
Cord section C$_{5-8}$	Normal	2.2 ± 0.1	
Control	Normal	3.0 ± 0.1	

suggesting a physiological role for endogenous corticosteroids in the timing of normal lung development. Identified biochemical processes stimulated by glucocorticoids include enzymes involved in surfactant synthesis (phosphatidic acid phosphatase, cholinephosphate cytidityltransferase, and possibly phosphatidylcholine remodeling enzymes), the overall rate of phosphatidylcholine synthesis from choline, membrane β-adrenergic receptor, and the rate of both glycogen synthesis and degradation (Brehier *et al.* 1977; Maniscalco, Shapiro 1983; Cheng *et al.* 1980; Rooney *et al.* 1976; Ballard 1979). The possible effect of glucocorticoids on the activity of other enzymes in phospholipid biosynthesis remains controversial (Ballard 1979). The response to corticosteroids is apparently quite specific, since two-dimensional thin layer chromatography analysis of synthetic rates indicated a hormone-induced stimulation for only 4 of 400 proteins resolved (Phelps, Giannopoulos 1983).

Studies in my laboratory have explored the mechanism of glucocorticoid action. Glucocorticoid receptors have been identified and characterized in fetal lungs of several species, including man (Ballard 1977; Ballard 1982). Receptor is also present in type II cells and fibroblasts of fetal lungs at a concentration similar to that for whole tissue, which is consistent with glucocorticoid effects in many different cell types of fetal lung (Ballard *et al*. 1978). These findings led to the proposal that glucocorticoids exert their effects in fetal lung by a mechanism involving *de novo* RNA and protein synthesis mediated by receptor.

Glucocorticoid receptor of lungs, as well as other tissues, contains sulfhydryl groups that must be in the reduced state for binding to occur (Granberg, Ballard 1977). When binding is assessed *in vitro* in cytosol preparations, there is a rapid loss of activity in the absence of sulfhydryl compounds, such as dithiothreitol or glutathione. Inactivation caused by air oxidation of receptor sulfhydryl groups is reversible when a sulfhydryl compound is added. A striking developmental change in this regard occurs in rat lungs; receptor from fetal tissue is relatively resistant to air oxidation, whereas complete inactivation occurs rapidly in cytosol prepared from adult tissue. This developmental change apparently reflects the concentration of endogenous sulfhydryl compounds, such as glutathione, in lung tissue. The possible signficance of this process *in vivo* is unknown, but reduction-oxidation of sulfhydryl groups may serve as an intermediary control mechanism in other hormone systems (Gilbert 1982).

Recent studies of fetal lungs in organ culture (Gross *et al*. 1980; Gross *et al*. 1983; Snyder *et al*. 1981; Maniscalco, Shapiro 1983) provided evidence for the involvement of the receptor system in glucocorticoid effects. In this system, fetal lung explants can be cultured in the absence of serum, and various hormonal responses can be studied. With cultured lungs from 24-day gestation fetal rabbits, the presence of dexamethasone produced about a doubling in the rate of choline incorporation into phosphatidylcholine (Gross et al. 1983). The response to corticosteroid has a lag phase of 6 to 12 hours and reaches optimal levels by 48 to 72 hours. Dexamethasone and cortisol are half-maximally effective in stimulating phosphatidylcholine synthesis at 0.7 and 6.8 nM, respectively. Other steroids with glucocorticoid activity also stimulate phosphatidylcholine synthesis, whereas those steroids without glucocorticoid activity are ineffective (Table 3). Furthermore, a close correlation exists between the concentration of steroid for half-maximal stimulation and the dissociation constant values for binding to receptor. This observation suggests a direct linear relationship between binding of corticosteroid to receptor and stimulation of phosphatidylcholine synthesis. Both actinomycin D, which inhibits RNA synthesis, and cycloheximide, an inhibitor of protein synthesis, block the stimulatory effects of glucocorticoids in this system. These findings are consistent with receptor mediation of the glucocorticoid effect in fetal lungs.

Considerable circumstantial evidence supports a role of endogenous corticosteroids in lung maturation. These include clinical correlations as well as experimental data (Ballard 1980; Ballard 1982). However, relatively little is known about the factors involved in the expression of the glucocorticoid influence. In some species such as the sheep, a close correlation is observed between total plasma cortisol concentration and parameters such as maximal lung volume and lung content of saturated phosphatidylcholine (Kitterman et al. 1979). For this species, cortisol increases markedly in the last few days before spontaneous delivery (Mescher et al. 1975). This increased corticosteroid production by the fetal adrenals appears to be the major factor regulating glucocorticoid effects in fetal sheep. In other species, however, the situation is less obvious. For example, in rabbits, rats, and mice, total corticosteroid levels decrease in late gestation when the lung is undergoing terminal differentiation. In these species, the concentration of plasma corticosteroid binding globulin (CBG) is also decreasing, which increases unbound plasma corticosteroid (Ballard 1979). The concentration of glucocorticoid receptors also increases during the last trimester (Giannopoulos et al. 1972; Ballard et al. 1983), and cortisone conversion to cortisol in fetal lung increases, which should increase the local concentration of available glucocorticoid (Nicholas et al. 1978; Smith et al.

Table 3. Corticosteroid Binding to Receptor and Stimulation of Phosphatidylcholine Synthesis

Steroid	Choline Incorporation (% of dexamethasone effect)	^{3}H-Dexamethasone Nuclear Binding (% inhibition)
Dexamethasone	100	88
Cortisol	96	62
Cortisone	92	41
Corticosterone	43	19
Dihydrocorticosterone	27	11
Progesterone	0	2
17α-Hydroxyprogesterone	0	0
17β-Estradiol	0	-6
Testosterone	0	-12

Data are mean values for experiments performed with 24-day gestation fetal rabbit lung in organ culture. Choline incorporation was assessed using 10^{-7} M steroid, and binding represents competition using 10^{-7} M unlabeled steroid and 10^{-8} M ^{3}H-dexamethasone. Data are from Gross et al. 1983.

1973; Smith et al. 1977). Thus, in these species multiple factors may be interacting to provide a glucocorticoid stimulus to lung development. Presumably, external agents affecting any of these biochemical processes could interfere with the timing of lung maturation. Also, glucocorticoids regulate developmental events in a number of tissues (Ballard 1979), and therefore, alterations in the glucocorticoid environment could produce multi-organ effects.

THYROID HORMONES

Exposure of the fetus to thyroid hormones produces effects in fetal lung similar to those observed with glucocorticoids, which include both morphological maturation as well as enhanced production of surfactant (Ballard 1980; Ballard 1982). Receptors for T_3 have been identified in the nuclei of animal and human fetal lungs (Lindenberg et al. 1978; Gonzales, Ballard 1981; Gonzales, Ballard 1982; Morishige, Uetake 1978). Receptor is also present in two cell lines that apparently originated from the type II alveolar cell (Lindenberg et al. 1978). In rabbit and human lungs, the average number of receptor sites per cell increases during gestation. For the rabbit, this increase shows a close temporal association with increased synthesis and secretion of surfactant between Days 26 and 28 of gestation (Table 4). The occupancy of nuclear receptors with endogenous thyroid hormones has been estimated from the level of binding at 37°C (measuring total receptor capacity) and 2°C (measuring only unoccupied receptor sites). Occupancy increases from 11% to 23% between Days 23 and 30. Combined with the increase in receptor number during this portion of gestation, a 3-fold increase in occupied receptor sites per cell in the fetal lung is calculated during late gestation (Table 4). These findings support an influence of endogenous thyroid hormones during the critical period of pulmonary differentiation.

Studies with thyroid hormones in organ culture have been similar to those for glucocorticoid. Recent data obtained in my laboratory indicate that T_3 stimulates choline incorporation into phosphatidylcholine and that the properties of this effect are consistent with a receptor-mediated mechanism (Ballard, Gonzales 1983). For example, half-maximal stimulation with T_3 occurs at 0.11 nM, and the K_D for T_3 binding is 0.13 nM. The activity of various thyroid hormone analogs for stimulating choline incorporation correlates closely with their binding affinity. The effect of T_3 is blocked in the presence of inhibitors of both RNA and protein synthesis. When both T_3 and dexamethasone are added to the cultures, the stimulation of choline incorporation is synergistic (Ballard, Gonzales 1983; Smith, Sabry 1983; Gross et al. 1980). This effect, which is still being characterized, provides direct evidence for multihormonal regulation of lung maturation.

Table 4. Occupancy of Nuclear T_3 Receptor by Endogenous Thyroid Hormones in Fetal Rabbit Lung

Gestational Age	T_3 Receptor Capacity (fmol/µg DNA)	Occupancy[a] (% of total sites)	Occupied Sites/Cell
21	0.267 ± 0.021	11.3 ± 4.7	110
25-26	0.313 ± 0.028	14.5 ± 4.2	165
28	0.384 ± 0.011	23.8 ± 1.4	330
31	0.273 ± 0.018	23.0 ± 2.8	227

[a] Mean ± SE.

Receptor capacity was assayed in isolated nuclei by dose-response studies of $^{125}I\text{-}T_3$ binding, and occupancy was estimated by the difference in binding at 37°C and 2°C as previously described (Gonzales, Ballard 1982). The number of occupied sites per cell is calculated from receptor capacity and occupancy. Data from Gonzales, Ballard 1982.

β-ADRENERGIC AGONISTS

These agents cause a prompt release of stored surfactant from type II cells and both decrease the secretion and stimulate reabsorption of fetal lung fluid (Ballard 1980; Ballard 1982). In addition, Kanjanapone *et al.* (1980) reported increased synthesis of phosphatidylcholine *in vivo* after isoxsuprine treatment. Effects of catecholamines have been characterized in isolated type II cells from adult lungs, and the properties of surfactant release with these agents are consistent with a specific β-adrenergic effect (Dobbs, Mason 1979). Catecholamine action is presumably mediated by membrane adrenergic receptors that activate adenylate cyclase to increase cyclic AMP levels. β-adrenergic receptors are present in fetal lungs of several species and their concentration increases during late gestation. In rats and rabbits, this increase occurs precociously with glucocorticoid treatment, both *in vivo* and in culture

(Maniscalco, Shapiro 1983; Cheng et al. 1980). This developmental change would increase the sensitivity of the fetal lung to β-adrenergic influence. In addition to affecting the number of receptors, glucocorticoids may also enhance the coupling of receptors to adenylate cyclase, which potentially provides a further amplification of the adrenergic influence. Endogenous catecholamines are probably important stimuli with regard to surfactant secretion and lung water before birth, but their precise physiological role has not been determined.

PROSTAGLANDINS

Before birth, mammalian fetuses make breathing movements only intermittently; with birth, the control of breathing changes because breathing occurs continuously. Breathing movements in fetal sheep, which have been studied more extensively, occur about 40% of the time and only when the fetal electrocorticogram shows low voltage, fast activity. Although a variety of exogenous stimuli influence these movements, the physiological factors that control fetal breathing movements are still uncertain. Recent experiments implicate prostaglandins as a possible physiological factor (Kitterman et al. 1983; Neutze et al. 1977; Kitterman et al. 1979). Administration of indomethacin or meclofenamate, inhibitors of prostaglandin synthesis, produces a striking increase in the incidence of breathing movements that occur even during high voltage, slow electrocortical activity, when movements are normally absent. With infusion of PGE_2, fetal breathing movements are eliminated. Infusion of $PGF_{2\alpha}$ and endoperoxide analogs have similar, but less profound effects.

Circulating concentrations of endogenous PGF_2 are significantly higher in fetal than in neonatal sheep (Clyman et al. 1980). Thus, a critical circulating concentration of PGE_2 that inhibits breathing movements may exist. If endogenous PGE_2 is an important factor in utero, then a decrease in circulating PGE_2 may contribute to the transition from the fetal condition of intermittent breathing movements to continuous breathing movements after birth. The site and mechanism of prostaglandin control of fetal breathing movements are not yet known. PGE_2 could act through chemoreceptors or directly on respiratory centers, possibly through interaction with norepinephrine.

HOROMONES AND ABNORMAL LUNG DEVELOPMENT

Because several classes of hormones are involved in lung development, hormonal imbalances could possibly produce abnormalities in lung development and/or function. Three common clinical conditions illustrate this possibility. While many cases of hypoplastic lung are secondary to mechanical obstruction of lung growth, the cause in other cases is not obvious. If the circulating levels of PGE_2 were increased due to a toxin, the incidence of fetal breathing movements would be decreased and lung growth could consequently be delayed.

Transient tachypnea of the newborn is a common condition that is apparently caused by delayed clearance of lung fluid. Based on our current understanding of hormonal mechanism and interaction, we could speculate that a decrease in either the circulating levels or catecholamine binding in lungs would delay reabsorption of lung fluid. Furthermore, low levels of circulating corticosteroids, or a delayed developmental increase in lung glucocorticoid receptors, could delay the increase in β-adrenergic receptors in fetal lung and also reduce the catecholamine influence.

Respiratory Distress Syndrome (RDS-Hyaline Membrane Disease) is a disease of surfactant deficiency that most commonly occurs in premature infants. However, it may occur in infants near term, and the degree of severity is variable at all gestational ages. Since the surfactant system is under multi-hormonal regulation, a toxic insult in any of the hormonal systems (production or binding) could increase the susceptibility to RDS. One interesting possibility for decreased glucocorticoid influence would be an effect of environmental toxin on the redox potential or level of sulfhydryl compounds in fetal lungs. Potentially, this could cause inactivation of glucocorticoid receptors with a subsequent delay in lung maturation.

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EFFECTS OF PRENATAL NITROFEN EXPOSURE ON POSTNATAL LUNG FUNCTION IN THE RAT

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INTRODUCTION

Prenatal exposure to the pre-emergent herbicide 2,4-dichlorophenyl-p-nitrophenyl ether (Nitrofen, TOK®*) has been shown to increase neonatal mortality in laboratory animals (Ambrose _et al_. 1971; Kimbrough _et al_. 1974; Stone 1978; Stone, Manson 1981; Costlow, Manson 1981). Many of the newborn die from respiratory distress within 24 h after birth. In addition, oral concentrations ranging from 20 to 250 mg/kg/d have been found to be very fetotoxic when given during Days 8 through 18 of gestation, the critical stages of organogenesis in the rat. Dose-related effects include decreased postnatal lung and body weights (Stone, Manson 1981; Costlow, Manson 1981; Kavlock, Gray 1983) and congenital structural anomalies of the diaphragm, heart, kidneys, and skeleton (Costlow, Manson 1981).

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*TOK® is a registered trademark of the Rohm and Haas Company. (Registered in U.S. Patent Office and in principal foreign countries.)

Since respiratory distress was the apparent cause of Nitrofen-induced neonatal mortality in the rat, Stone (1978) speculated that Nitrofen might have a direct effect on lung maturation. However, results from these studies (Stone 1978; Stone, Manson 1981) failed to show interference with glucocorticoid or pulmonary surfactant synthesis at Nitrofen exposures of 20 to 50 mg/kg/d during Days 8 through 18 of gestation. Follow-up studies by Costlow and Manson (1981) identified the heart and diaphragm as the target organs in Nitrofen-induced neonatal death.

Recent investigations with Nitrofen have demonstrated that surviving offspring with no apparent morphological alterations at birth develop intraorbital defects (Gray *et al*. 1982) and delayed morphological and biochemical differentiation of the kidney (Kavlock *et al*. 1982) during the postnatal period. Nitrofen-induced hydronephrosis has previously been demonstrated at high oral doses (Costlow, Manson 1981). However, Kavlock and Gray (1983) found impairment of kidney function in the absence of gross morphological changes at low doses of Nitrofen given during Days 8 through 16 of gestation. It is possible that prenatal exposure to Nitrofen could cause functional impairment of other organ systems, particularly the lung. The purpose of this study was to determine if low levels of Nitrofen given during the critical stages of embryogenesis would cause alterations in postnatal lung growth and development in the rat.

MATERIALS AND METHODS

Animals

Timed pregnant Fischer-344 rats (CDF, F-344, Crl BR, Charles River Laboratories, Wilmington, Massachusetts) were used for this study because of previous laboratory experience with lung function measurements during the postnatal period of lung growth and development (Raub *et al*. 1983a). The day that sperm was found in the vaginal smear was considered Day 1 of pregnancy. The animals were received on Day 2 of pregnancy and housed individually in plastic cages with pine shaving for bedding material. Food and water were provided *ad libitum*.

Exposure

The primiparous rats were randomly assigned to control or experimental groups. Exposed animals (TOK®) were given Nitrofen (15 mg/kg/d in 0.1 ml/100 g BW of corn oil) by gavage during Days 10 through 13 of gestation. Control animals (CNT) were given the corn oil alone. This dose was chosen because previous studies in this laboratory indicated it would result in low neonatal mortality with gradual decreases in lung weight during the postnatal period in the absence of a gross body weight effect (unpublished results). One day after normal delivery, the newborn pups were pooled by sex and then randomly reassigned to litters with a maximum of six pups per litter. Only male rats were used for subsequent measurements of pulmonary function and morphology. The pups were weaned at postnatal Day 21 and given water and food *ad libitum*.

Lung Function

Measurements of pulmonary function were obtained at 3 and 6 weeks of age. The techniques used for measurement of lung function were similar to those previously reported in small laboratory animals (Raub *et al*. 1982; Raub *et al*. 1983b). Rats were anesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal injection, tracheostomized, and placed in a whole-body pressure plethysmograph for measurement. Lung volume was obtained from changes in plethysmographic pressure measured by a differential pressure transducer (Validyne, MP-45, Northridge, California) referenced to a chamber of equal volume and open to atmospheric pressure through a high impedance pathway. Airway pressure was measured by a differential pressure transducer (Validyne, MP-45, Northridge, California) referenced to the plethysmograph providing the pressure difference across the total respiratory system.

After a short equilibration period of resting breathing, breathing frequency and tidal volume were recorded. The airway was occluded at end-expiration, and end-expiratory volume was calculated using Boyle's Law by recording airway pressure and plethysmographic pressure as the animal attempted to inspire against the closed airway. The animals were paralyzed with succinylcholine chloride (0.5 mg/kg) by intraperitoneal injection and maintained on a small animal respirator (Model 5255, Ealing Corp., Natic, Massachusetts). Pressure-volume (P-V) curves were obtained by inflating and deflating the lungs with a syringe pump. Tangent compliance was measured along the deflation limb of the P-V curve by fitting the data to an exponential equation. Vital capacity was measured between a pressure difference of -15 cm H_2O and +30 cm H_2O. Single-breath diffusing capacity for carbon monoxide and residual volume were measured by gas dilution techniques previously described from this laboratory (Takezawa _et_ _al_. 1980).

The plethysmograph used for these studies was constructed with a low dead space valve that allowed switching between room air (79.6% N_2) and 100% oxygen. By continuously sampling airway N_2 concentrations (Med Science 505, Med Science Electronics, Inc., St. Louis, Missouri), multiple-breath N_2 washouts were performed using mechanical ventilation at a fixed volume and frequency. Volume and airway N_2 signals during the maneuver were recorded by a PDP-8M minicomputer system (Digital Equipment Corporation, Maynard, Massachusetts) for later analysis. The natural log of end-expiratory N_2 concentrations was plotted against breath number and reported as the N_2 slope. Relative differences in the intrapulmonary distribution of ventilation were evaluated by comparing the normalized first moment ratio M_1/M_0 of the lung N_2 washout (Saidel _et_ _al_. 1975).

All measurements of pulmonary function were performed twice in each animal and then averaged. The effects of Nitrofen were evaluated statistically by comparing the weight-adjusted means of each pulmonary function measurement using a one-way analysis of covariance. Significant differences were indicated at the 0.05 probability level.

Morphology

A separate group of rats was used to evaluate the histopathological changes resulting from Nitrofen exposure. The animals were sacrificed at 3 and 6 weeks of age with an intraperitoneal injection of sodium pentobarbital solution (75 mg/kg) followed by exsanguination. The liver, kidneys, and testes were removed and weighed. Sections were fixed in 10% formalin. The lungs were fixed _in_ _situ_ by instillation of a 2% cacodylate-buffered glutaraldehyde solution at a pressure of 30 cm H_2O using methods described previously (Hayatdavoudi et al. 1980). Paraffin sections were prepared and stained for evaluation by light microscopy.

RESULTS

General

Average litter size ($\bar{X}$ ± SD) was not significantly altered by Nitrofen exposure. Control (CNT) and exposed (TOK®) litters had 8.0 ± 1.6 and 7.8 ± 0.9 pups, respectively. However, average pup weight ($\bar{X}$ ± SD) was significantly reduced ($p < 0.05$) at birth in the animals exposed to Nitrofen. The TOK® pups weighed 4.85 ± 0.21 g compared to 5.20 ± 0.19 g for the CNT group. Dying pups were not collected for evaluation in this study. In previous experiments using Sprague-Dawley rats (Kavlock, Gray 1983) Nitrofen reduced pup viability primarily because of diaphragmatic hernias at doses of 12.5 and 25 mg/kg/d administered during Days 8 through 16 of gestation. A few of the surviving TOK® pups were noticeably smaller in size and most of the exposed animals could be identified by the presence of porphyrin rings around the eyes, indicating previously described abnormal secretions of the Harderian glands (Gray et al. 1982).

Lung Function

The effects of Nitrofen exposure on lung and body weight are shown in Table 1. There were no significant differences in wet or dry lung weights at either postnatal age. As noted above, some of the TOK® pups were noticeably smaller in size, but the average body weight was not significantly different from control. Small differences in animal weights were accounted for by adjusting the pulmonary function values for body weight using a one-way analysis of covariance.

Patterns of ventilation are shown in Table 2. There was a 10% decrease ($p < 0.01$) in the tidal volume of TOK® rats at 6 weeks of age, which was only partially compensated for by a slight but nonsignificant increase in breathing

Table 1. Summary of Lung Weights and Body Weights in Rats Prenatally Exposed to Nitrofen[a]

Treatment Group[b]	n	Body Weight (g)	Wet Lung Weight (g/kg)	Dry Lung Weight (g/kg)
CNT-3WK	15	34.9 ± 1.2	10.03 ± 0.39	2.16 ± 0.07
TOK®-3WK	15	33.6 ± 0.9	9.67 ± 0.24	2.08 ± 0.05
CNT-6WK	15	126.8 ± 2.2	5.71 ± 0.17	1.34 ± 0.03
TOK®-6WK	15	121.7 ± 3.4	5.99 ± 0.13	1.34 ± 0.04

[a] All data are reported as the mean ± standard error of the mean.

[b] Control (CNT) rats given corn oil and rats given 15 mg/kg/d Nitrofen (TOK®) during Days 10 through 13 of gestation were evaluated at 3 and 6 weeks of age.

frequency. Minute ventilation was slightly reduced but not significantly different from control rats in that age group.

Changes in lung volume and compliance are illustrated in Fig. 1. There were no significant differences at 3 weeks of age. However, by 6 weeks of age, TOK® rats had a 7.3% decrease ($p < 0.01$) in vital capacity (VC) and a 5.7% decrease ($p < 0.05$) in total lung capacity (TLC). Quasi-static lung compliance decreased 8.2% ($p < 0.01$) from 0.353 ± 0.005 (SE) in CNT rats to 0.324 ± 0.005 (SE) in TOK® rats. Significant changes in the distribution of ventilation were also measured in TOK® rats at 6 weeks of age (Fig. 2). The N_2 washout slope increased 11.2% ($p < 0.01$) and the normalized moment ratio increased 6.3% ($p < 0.05$). There was a slight but insignificant ($p = 0.08$) decrease in diffusing capacity in the same group of animals (Fig. 3).

Table 2. Summary of Spontaneous Ventilation in Rats Prenatally Exposed to Nitrofen[a]

Treatment Group[b]	n	Frequency (breaths/min)	Tidal Volume (ml)	Minute Ventilation (ml/min)
CNT-3WK	15	83 ± 4	0.34 ± 0.01	28.4 ± 1.2
TOK®-3WK	15	92 ± 4	0.32 ± 0.01	29.4 ± 1.2
CNT-6WK	15	90 ± 4	0.90 ± 0.02	81.0 ± 2.9
TOK®-6WK	15	96 ± 4	0.81 ± 0.02[c]	77.8 ± 2.9

[a] All data are reported as the mean ± standard error of the mean.

[b] Control (CNT) rats given corn oil and rats given 15 mg/kg/d Nitrofen (TOK®) during Days 10 through 13 of gestation were evaluated at 3 and 6 weeks of age.

[c] Significance at the $p < 0.01$ level.

Morphology

Organ-to-body weight data are summarized in Table 3. There were no significant effects attributable to Nitrofen. Histopathologic findings also revealed no significant microscopic changes in the lungs, kidneys, liver, or testes. Two of the eight TOK® rats studied at 3 weeks of age had diaphragmatic hernias with portions of the liver protruding into the thorax. No significant lesions were observed in these animals.

DISCUSSION

Results of the current study indicate that prenatal exposure to the herbicide Nitrofen causes functional impairment of the lung during postnatal growth and development. The lungs of Nitrofen-exposed animals at 6 weeks of age were smaller in total lung volume and less compliant. There was also an inhomogeneous distribution of

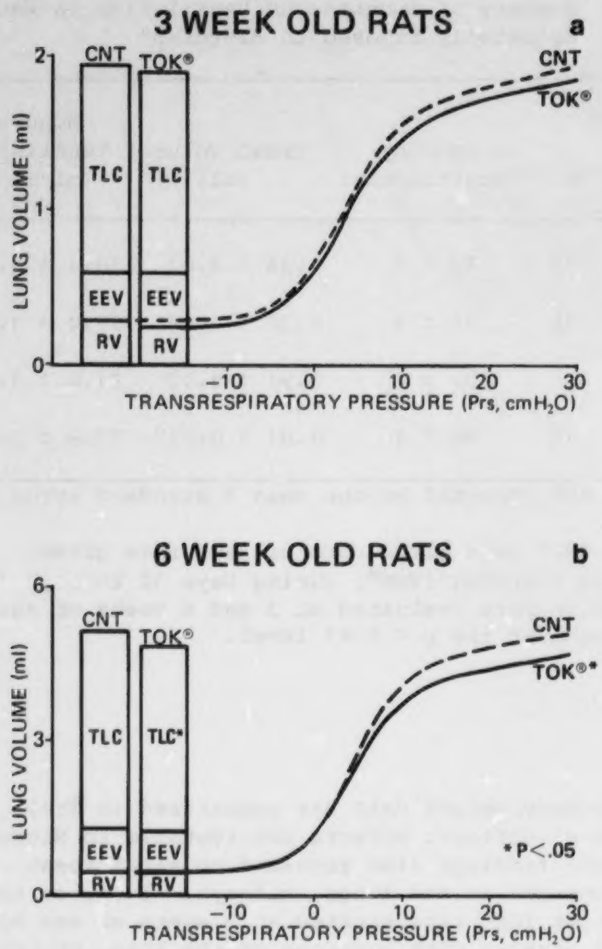

Fig. 1. Lung compartment volumes and lung deflation pressure-volume relationships in (a) 3-week-old and (b) 6-week-old Nitrofen (TOK®)-exposed rats compared to controls (CNT). TLC = total lung capacity; EEV = end-expiratory volume; RV = residual volume.

alveolar ventilation. Furthermore, this impairment becomes more evident as the animals grow to maturity.

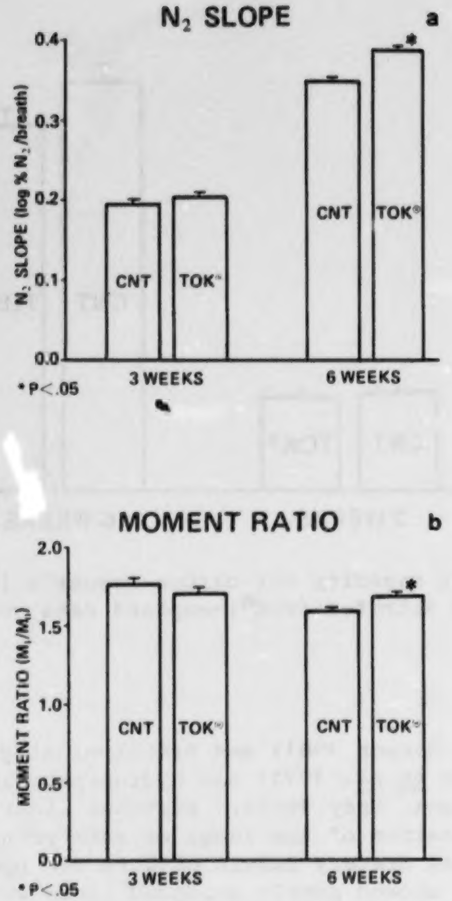

Fig. 2. Intrapulmonary gas distribution and mixing was characterized by (a) the multiple-breath nitrogen washout slope (N_2 Slope), and (b) the ratio of the 1st to 0th moment (M_1/M_0) in 3- and 6-week-old Nitrofen (TOK®)-exposed rats compared to controls (CNT).

The lack of morphological effects on the liver, kidney, and testes is consistent with previous dose-response studies conducted at low concentrations of Nitrofen (Ambrose *et al.* 1971; Kavlock, Gray 1983). Higher doses have resulted in structural malformations of the diaphragm, heart, and

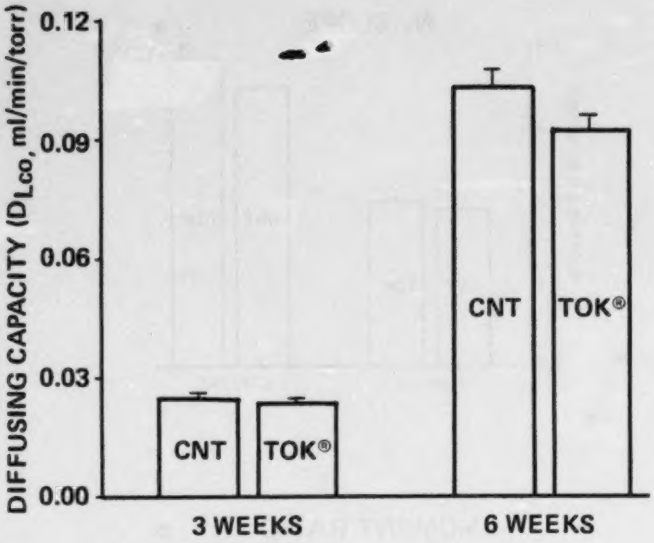

Fig. 3. Diffusing capacity for carbon monoxide (D_{LCO}) in 3- and 6-week-old Nitrofen (TOK®)-exposed rats compared to controls (CNT).

kidneys (Costlow, Manson 1981) and histological diagnosis of hepatosis (Ambrose et al. 1971) and hydronephrosis (Costlow, Manson 1981; Kavlock, Gray 1983). Previous light microscopic examination of the lungs of rats prenatally exposed to maternal dietary levels of 0 to 500 ppm Nitrofen (0 to 46 mg/kg/d) showed poorly expanded lungs with immature alveolar epithelium (Kimbrough et al. 1974; Stone, Manson 1981). Electron microscopy on these lungs revealed extensive fibrosis with inflammation and proliferation of epithelial cells in animals dying within 2 weeks following birth but only subtle ultrastructural differences in surviving animals (Kimbrough et al. 1974).

Ventilation inhomogeneity has been detected in human clinical medicine with the use of similar methods. Normal smokers, and to a greater extent, patients with asthma, diffuse interstitial lung disease, and chronic obstructive lung disease all have measurable increases in N_2 slope and M_1/M_0 (Fleming et al. 1980; Saniie et al. 1979). It would be difficult to associate the changes in Nitrofen-exposed

Table 3. Organ-to-Body Weight Ratios in Rats Prenatally Exposed to Nitrofen[a]

Treatment Group[b]	n	Body Weight (g)	Liver (g/kg)	Kidney (g/kg)		Testes (g/kg)	
				Left	Right	Left	Right
CNT-3WK	9	42.1 ± 1.8	41.43 ± 1.09	6.11 ± 0.17	6.46 ± 0.28	3.26 ± 0.13	3.09 ± 0.15
TOK®-3WK	8	41.1 ± 1.1	41.79 ± 1.50	6.32 ± 0.12	6.77 ± 0.12	3.35 ± 0.11	3.29 ± 0.14
CNT-6WK	8	141.0 ± 2.2	48.48 ± 0.91	4.72 ± 0.04	4.84 ± 0.10	5.87 ± 0.10	5.69 ± 0.12
TOK®-6WK	8	130.0 ± 3.0	48.01 ± 0.64	4.84 ± 0.11	5.06 ± 0.16	5.73 ± 0.11	5.71 ± 0.14

[a] All data are reported as the mean ± standard error of the mean.

[b] Control (CNT) rats given corn oil and rats given 15 mg/kg/d Nitrofen (TOK®) during Days 10 through 13 of gestation were evaluated at 3 and 6 d of age.

rats with any of the clinical diseases, since results of the histopathological evaluation were negative. Similarly, it would be difficult to characterize these lungs as hypoplastic, defined by Page and Stocker (1982) as incomplete development of the lung, because morphometric measurements were not performed in this study. There were no indications of changes in pulmonary alveolarization, commonly identified by the size and number of alveoli, that could be detected by the light microscopy performed.

Nevertheless, there are striking similarities between the involvement of multiple organ systems in Nitrofen toxicity of rats and lung, diaphragm, and kidney anomalies associated with pulmonary hypoplasia in children (Page, Stocker 1982). Both may be caused by an interference of normal development during a critical stage of embryogenesis (Costlow, Manson 1981; Swischuk _et al_. 1979; Page, Stocker 1982). Extensive impairment of lung growth, identified morphometrically by reduced airway, arterial, and alveolar development, has been noted in infants dying from diaphragmatic hernias (Kitagawa _et al_. 1971). Speculation that further alveolar multiplication during growth was unlikely to make up these differences following successful repair of the hernia was confirmed by Thurlbeck _et al_. (1979). They demonstrated impaired lung growth despite restoration of lung volume and also suggested that a lack of arterial development in the lung may be involved. Congenital malformations of the heart have also been shown to be associated with abnormal pulmonary vascularization and alveolarization in children with tetralogy of Fallot who commonly have smaller lungs and body size (Hislop, Reid 1973). It is interesting to note that the heart has been shown to be a target organ in Nitrofen-induced neonatal mortality in rats (Costlow, Manson 1981). This suggests the possibility of more subtle changes in pulmonary vascular development at lower concentrations.

There have been very few studies designed to evaluate prenatal factors affecting lung growth and development in laboratory animals. Karotkin _et al_. (1976) reported that maternal treatment with phenobarbital has an inhibitory effect on surfactant synthesis and release by the fetal rabbit lung. Nitrofen has not been shown to have an inhibitory effect on the surfactant system in rats despite having a generalized inhibitory effect on lung maturation (Stone 1978; Stone, Manson 1981) and overall body growth

(Kavlock et al. 1982). More recently, retinyl acetate given to pregnant rats by gavage during Days 15 through 19 of gestation produced changes in lung compliance in pups at 3 d of age (Newman, Johnson 1981). These changes were reportedly associated with septal wall thickening and marked atelectasis with adjacent areas of lung overinflation, effects previously noted in rat pups exposed to Nitrofen (Kimbrough et al 1974; Stone, Manson 1981).

In conclusion, previous studies with the herbicide Nitrofen have suggested that examination of fetuses at the time of parturition may not be sufficient indications of fetotoxicity since other effects can appear during the postnatal period (Gray et al. 1982; Kavlock, Gray 1983). Results from the current study suggest a delayed or impaired functional development in the lungs of rats prenatally exposed to Nitrofen. These changes appear to become more evident as the animal ages. It may be necessary to assess physiological function from birth through maturity in order to completely examine the effects of prenatal exposure to a potentially toxic substance. The Nitrofen-exposed rat may be an ideal animal for studying the lung maturation process during these stages of development, as suggested previously by Stone (1978). It may also be a useful model of lung hypoplasia if future studies are able to demonstrate changes in lung alveolarization.

SUMMARY

The herbicide Nitrofen was administered by gavage to pregnant F-344 rats during Days 10 through 13 of gestation. Postnatal lung function was measured in male progeny at 3 and 6 weeks of age. There were no differences in body weight or wet and dry lung weights between control and Nitrofen-exposed rats in either age group. Nitrofen produced no observable effects on lung function at 3 weeks of age. However, by 6 weeks of age the Nitrofen-exposed animals had significant decreases in tidal volume ($p < 0.01$), vital capacity ($p < 0.01$), total lung capacity ($p < 0.05$), and quasi-static lung compliance ($p < 0.01$). There was also a mild ventilation inhomogeneity, as indicated by significant increases in the nitrogen washout slope ($p < 0.01$) and the moment ratio ($p < 0.05$). Histopathology of lung, liver, kidney, and testes was not significantly altered by Nitrofen exposure. These data

suggest that prenatal Nitrofen exposure may have an effect on postnatal lung maturation in the rat and could potentially be useful as a model of pulmonary hypoplasia.

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PARACRINE REGULATION OF LUNG GROWTH AND MATURATION: THE SUBSTRATE OF NORMAL FUNCTIONAL DEVELOPMENT

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INTRODUCTION

The development of complex organs such as lungs must require that the various cell types be in close communication during development. Several recent investigations have examined this possibility and suggest that such paracrine phenomena do occur during development. Whether these phenomena occur during later life is unclear, but they might be expected to participate in later reparative roles.

LUNG MATURATION

Glucocorticoids have long been known to influence the maturation of fetal organs, particularly those of endodermal origin, such as gut (Moog 1953), liver (Cake *et al*. 1981), pancreas (Rall *et al*. 1977), and salivary glands (Kumegawa *et al*. 1978). Relatively recently, this knowledge has been applied to the developing lung, another endodermally derived organ (Buckingham *et al*. 1968), and an extensive literature has been accumulated. Despite the more recent nature of such investigations, this is the first organ system in which such knowledge has been clinically applied (Collaborative Group on Antenatal Steroid Therapy 1981).

As with many developing organs, the appearance of structure (organogenesis) in the embryonic lung is strongly dependent on bidirectional interactions between epithelium and mesenchyme (Smith, Fletcher 1979). The initial lung bud develops from the ventral surface of the endodermal foregut and gives rise to the epithelium of the respiratory tract. As it grows and branches repeatedly, it is surrounded by a loose mesenchyme, which will ultimately give rise to the pulmonary interstitium, smooth muscle, cartilage, and endothelium. The branching pattern of the endoderm is strongly influenced by the surrounding mesenchyme; organ explants of the lung bud (endoderm plus mesenchyme) continue to branch in vitro, but if the mesenchyme is removed, branching ceases (Wessells 1970). This effect of the mesenchyme is organ-specific and also region-specific. Bronchial mesenchyme will induce the lateral tracheal epithelium, which normally does not bud, to bud and conversely, paratracheal mesenchyme will inhibit budding of the more peripheral airway epithelium (Wessells 1970).

In later fetal lung development, when hormonal regulation is occurring, epithelial-mesenchymal interactions continue to occur more subtly through the modulation of some hormonal responses (Smith, Fletcher 1979). Glucocorticoids stimulate surfactant synthesis by alveolar epithelial type II cells in organ cultures, or in mixed monolayer or organotypic cultures of fetal lung tissue (Ballard 1983; Gross 1983), each of which contains cells derived from the various germ cell layers. In contrast, in studies using cloned human fetal alveolar epithelial type II cells (Tanswell, Smith 1980), the glucocorticoid effect was found to be very much attenuated (Smith 1978). However, if these cells are recombined with fetal lung fibroblasts, full glucocorticoid stimulation of surfactant synthesis is again seen. Exposure of the type II cells to conditioned medium from glucocorticoid-treated fetal lung fibroblasts also results in full responsivity (Smith 1978). These observations appear to be due to fetal lung fibroblast production, in response to glucocorticoid, of fibroblast-pneumonocyte factor (FPF), a low molecular weight, heat-stable polypeptide (Smith 1978). The production of FPF by fibroblasts appears to be organ-specific (Smith 1978), but not species-specific (Smith 1981). Its production by fetal rat lung fibroblasts is maximal in cells isolated at the time of maturation of the surfactant system (Smith 1981). FPF, produced by human fetal lung fibroblasts and partially purified by column chromatography, has been shown to accelerate lung maturation in the fetal rat (Smith 1979). FPF is present in human amniotic fluid (Seybold, Smith 1980). In keeping with its polypeptide nature, FPF has recently been shown to increase intracellular 3'5'-cyclic AMP levels in alveolar type II cells (Smith, Sabry 1983). The time course of glucocorticoid effect on FPF production, intracellular cyclic AMP accumulation, and surfactant synthesis in mixed (fibroblast-epithelial) cell cultures isolated from fetal rat lung is consistent with our belief that the major effect of glucocorticoid on surfactant production is mediated by FPF production by the mesenchyme, followed by FPF stimulation of synthesis in the alveolar epithelium (Smith, Sabry 1983). We have recently succeeded in raising monoclonal antibodies to active sites in FPF that, when injected into chick embryos, arrest lung maturation (Post _et al_. 1983). This observation implies that FPF is important in lung maturation under baseline conditions. Interestingly, despite an intensive immunization protocol, both anti-FPF monoclonal antibodies that have been studied in detail are of the immunoglobulin M class (Post _et al_. 1983), a fact that is consistent with the highly conserved nature of the peptide (Smith 1981).

Thyroid hormones also appear to regulate fetal lung maturation (Ballard 1983). However, their action appears to be different from, although complementary to, the action of glucocorticoids. Thyroid hormones do not induce FPF production, but appear to act directly on the epithelium to enhance responsiveness to FPF, with respect to both cyclic AMP generation and to surfactant synthesis (Smith, Sabry 1983).

Recent morphological observations are compatible with the role of epithelial-mesenchymal interactions in this process. Marin _et al_. (1982) studied the cellular relationships of the alveolar type II cell during lung maturation in the rat. In late fetal life, when the surfactant system is glucocorticoid-responsive, 100% of the type II cells are in direct association with fibroblasts. At birth, after full expression of alveolar type II cell differentiation, this figure is reduced to about 55% and falls still further postnatally, with a concomitant increase in associations between epithelium and endothelial cells, which facilitate gas exchange. Leung _et al_. (1980) examined organ cultures of developing rat lungs at various gestational ages. At all stages, both epithelial cells and fibroblasts take up labelled prednisolone. However, this glucocorticoid only accelerates lung maturation in Day 19 and 20 explants. On these same days, the basement membrane is breached and epithelial cells and interstitial cells come into very close contact (Leung *et al*. 1980).

A very similar change to that noted above occurs in the appearance of close epithelial-mesenchymal contacts in another foregut derivative, the duodenum, at the time the duodenal epithelium becomes responsive to glucocorticoids (Mathan *et al*. 1972). Preliminary evidence suggests a dependence of duodenal mesenchyme for glucocorticoid responsiveness of duodenal epithelium (Smith, Zinman 1982). In another foregut derivative, Dow *et al*. (1983) have described fibroblast-hepatocyte factor (produced in liver mesenchyme in response to glucocorticoid), which stimulates tyrosine aminotransferase activity in the chick embryo hepatocyte. Fibroblast-hepatocyte factor has been shown to be antigenically different from FPF (Dow *et al*. 1983).

LUNG GROWTH

A number of prenatal or postnatal insults can alter lung growth (Langston 1983; Wohl 1983). The study of lung growth is complicated by a large number of changes occurring *pari passu* with this process, so that a number of correlations might be expected with accretion of lung cells. Many of the correlations will not represent cause and effect relationships. However, if growth can be experimentally manipulated, then associations that persist under these conditions are much more likely to be of regulatory significance. For many years, it has been recognized that removal of one lung results in compensatory growth of the contralateral lung (Thurlbeck 1975). Because growth hormone appears to play a permissive role in this response (Brody, Buhain 1973), recent studies have focused on a possible role for somatomedins in this process. The somatomedins are a family of low molecular weight peptides; their concentrations in serum are growth-hormone dependent and they are mitogenic *in vitro* (Hall *et al*. 1975). Following pneumonectomy in the rabbit, activity that can increase alveolar type II cell growth *in vitro* appears in the serum (Smith *et al*. 1980). Because this serum activity is present in the high molecular weight fraction but is dissociable under acid conditions (features similar to the somatomedins), further studies were undertaken in the rat.

In rats, pneumonectomy or nephrectomy results in organ-specific hypertrophy of the contralateral organ (Sosenko et al. 1981). Despite this, serum somatomedin-C (SMC) levels (assayed by Dr. A. Joseph D'Ercole, University of North Carolina, Chapel Hill) were depressed by the surgical procedures and were not different between sham-operated animals and animals that had their lungs or kidneys removed (Sosenko et al. 1982). However, in regenerating kidney or lung tissues (Stiles et al. 1983), SMC levels were significantly elevated. These increases, in the absence of elevated serum levels, strongly suggest a paracrine mechanism for regenerative organ growth. In hypophysectomized rats there is also a tendency toward elevated kidney tissue SMC levels (Stiles et al. 1983), suggesting that growth hormone, while permissive, may not be the primary trigger for this response.

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EFFECTS OF MATERNAL CADMIUM EXPOSURE IN THE RAT ON PRENATAL MATURATION OF THE PULMONARY SURFACTANT SYSTEM

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INTRODUCTION

We are interested in detecting deficits in physiological function that are induced prenatally because we believe that 1) functional abnormalities are undetectable by routine teratological screening methods and therefore, these insidious manifestations of developmental toxicity are escaping our attention; and 2) functional deficits are more sensitive indicators of developmental toxicity than gross anatomical malformations (Grabowski, Daston 1983).

Experimentally induced developmental lung defects are infrequently observed in routine teratology screens. Thus, teratologists have paid little attention to the developing lung, which is clinically illogical because developmental pulmonary anomalies cause the greatest number of human neonatal deaths (Schaffer, Avery 1977). The most important of these anomalies is a functional deficit: insufficient prenatal maturation of the pulmonary surfactant, which generally results in respiratory distress syndrome (RDS) postnatally. RDS is the leading cause of neonatal death in the U.S. (Farrell, Wood 1976) and other developed nations (Reed _et_ _al_. 1978). It has been established for almost 25 years that an immature pulmonary surfactant system causes neonatal RDS (Avery, Mead 1959), and pharmacological approaches to accelerate prenatal surfactant maturation have been applied for more than a decade (Smith, Bogues 1980).

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Despite this, the overall percentage of neonatal deaths in the U.S. attributed to RDS has risen from 14.7% in 1968 to 17.5% in 1978 (Perelman, Farrell 1982).

Neonatal RDS in humans has traditionally been thought of as a problem of prematurity. I was interested in whether environmental agents were capable of producing a deficit in prenatal pulmonary surfactant development resulting in postnatal RDS. The nonessential heavy metal Cd had been previously shown to cause lung hypoplasia in the fetal rat (Chernoff 1973). Since the developing lung appears to be a specific target organ for Cd, it was a good candidate for altering pulmonary surfactant development as well.

METHODS

Animals

Long Evans rats were used throughout this study. Virgin females in estrus were caged overnight with males, and the following morning was designated gestation day (GD) 0. The rats were maintained in a constant environment (25.5 ± 1.0°C, 50 ± 5% relative humidity, 12 hours light/day), and fed commercial lab chow and tap water *ad libitum*.

Pregnant rats received daily subcutaneous (sc) injections of 0 or 8 mg/kg $CdCl_2$ in 0.9% NaCl on GD 12 to 15. Controls were sacrificed from GD 17 to 21 (term). Cd-treated rats were sacrificed from GD 18 to 22, since we found that parturition is delayed by about 24 hours in this group. In all cases for the biochemical and functional procedures described below, all the lungs of each litter were pooled. Thus, each data point reflects the litter mean, and the litter is the statistical unit.

Pulmonary Surfactant Phospholipid Accumulation in the Fetus. Fetal lungs were excised, washed in ice-cold saline, and homogenized. Phospholipids were extracted from the homogenate by the method of Folch *et al*. (1957). Individual phospholipids were separated by thin layer chromatography (TLC). Phosphatidylcholine (PC) and sphingomyelin (SPH) were the most abundant phospholipids, and these were quantitated by measuring the spot area on the chromatogram with a compensating polar planimeter. More complete methods can be found in Daston and Grabowski (1979).

Incorporation of [^{14}C]Choline into Fetal Lung Slices In Vitro. Synthesis rates of PC, SPH, and disaturated PC (DSPC) were determined by measuring the incorporation of (Me-^{14}C)choline (New England Nuclear, Boston, MA) into these phospholipids. Tissue slices (0.5 mm thick) were prepared from fetal lungs and incubated at 37°C in the presence of 1 µCi [^{14}C]choline for 1 hour. The incubation was stopped by placing the incubation flasks into ice. Phospholipids were extracted and separated by TLC as described above, and the individual phospholipid spots were counted for radioactivity. A more complete description of these methods is available in Daston (1982).

Pulmonary Glycogen Content. Glycogen was extracted from fetal lung homogenate using trichloroacetic acid and quantified by the anthrone reaction (Seifter et al. 1950). This procedure is more fully presented in Daston (1982).

Ultrastructural Development of Alveolar Epithelium. The caudal portion of fetal lung was processed for observation by transmission electron microscopy. In addition to qualitative observations of cytodifferentiation, we attempted to quantify the percentage of lamellar bodies and type II cells (characterized by the presence of at least one lamellar body) present in the alveolar epithelium. A more complete description of these procedures is presented in Daston (1981a).

Dose-Response Study. Progressively lower doses of $CdCl_2$ were used to determine whether pulmonary surfactant maturation could be affected in the absence of maternal or fetal toxicity. Pregnant rats received daily sc injections of 0, 1, 2, or 4 mg/kg $CdCl_2$ on GD 12 to 15, and phospholipids were extracted and separated as described previously (Daston 1981b).

RESULTS AND DISCUSSION

A daily dose of 8 mg/kg $CdCl_2$ on GD 12 to 15 resulted in an almost 7-fold increase in fetal mortality (54% vs. 8% in controls). The body weight of the treated fetuses was 20 to 30% lower than controls from GD 18 to 22. The Cd treatment decreased fetal lung weight by 35 to 50% during this period. The relative lung weight (lung/body weight ratio) was also significantly decreased in the Cd-treated fetuses, confirming Chernoff's (1973) earlier finding and demonstrating that the decrease in lung weight was substantially greater than the body weight retardation (Daston, Grabowski 1979).

Pulmonary Surfactant Maturation

SPH and PC were the most abundant phospholipids in the fetal lungs. Data on the developmental profile for these phospholipids are presented in Table 1. Pulmonary SPH content increased at a constant and equivalent rate in both control and treated groups. Accumulation of SPH was decreased in the treated group, but this was only on GD 18 and 19. Parturition was delayed by 24 hours in the Cd group, and the extra day of gestation provided sufficient time for the lung SPH content of the Cd group to reach the level of the control group at term.

PC, the principal component of pulmonary surfactant, also accumulated at a constant rate in controls and treated fetuses until the last day of gestation. Between GD 20 and 21 in controls, pulmonary accumulation of PC increased sharply, probably as the final prenatal maturation step for the pulmonary surfactant. The Cd-treated fetuses did not have this increase in PC accumulation at the end of gestation and at term had only 77% as much pulmonary PC as the term controls.

From these results, maternal Cd treatment appears to induce a deficit in fetal pulmonary surfactant development. However, although the absolute values for PC were lower in the Cd-treated fetuses than in controls, the PC concentrations were actually slightly higher in the Cd fetuses because the lung weight was so greatly reduced in this group. Thus, although the developmental profile for PC was different in the Cd group, and these fetuses did not have the surge in PC accumulation at the end of gestation, it is unclear whether this was caused by an actual effect on pulmonary surfactant synthesis or simply to the morphological effect on the lung.

Table 1. Effects of 8 mg/kg/day $CdCl_2$ on Fetal Lung Morphological and Biochemical Parameters[a]

Treatment and Gestation Day	Number of Litters	Fetal Weight (g)	Lung Weight (mg)	Lung PC (μg)	Lung SPH (μg)	Lung PC/Lung Weight (μg/mg)	Lung SPH/Lung Weight (μg/mg)	Lung Glycogen/ Lung DNA (μg/μg)
0 mg/kg								
17	4	0.94±0.02[b]	20.1± 2.0	25± 6	12± 2	1.2±0.2	0.6±0.1	--
18	10	1.52±0.02	39.6± 1.4	57± 6	25± 2	1.4±0.1	0.6±0.1	1.2±0.1
19	10	2.41±0.11	78.7± 4.7	95± 8	55± 7	1.2±0.1	0.7±0.1	2.5±0.3
20	9	4.33±0.24	142.9± 8.8	119± 8	60± 5	0.9±0.1	0.4±0.1	2.9±0.6
21	10	5.48±0.21	163.8± 5.1	214±16	80± 8	1.3±0.1	0.5±0.1	0.9±0.3
8 mg/kg								
18	5	1.14±0.04[c]	20.3± 3.2[c]	37± 9	16± 4[c]	1.8±0.4	0.8±0.2	--
19	11	1.95±0.08[c]	51.3± 5.5[c]	64± 7[c]	36± 4[c]	1.3±0.1	0.7±0.1	1.9±0.2[c]
20	7	3.04±0.16[c]	73.5± 7.3[c]	99±12	48± 6	1.4±0.2[c]	0.7±0.1	1.7±0.1[c]
21	8	4.14±0.23[c]	102.6±10.6[c]	137±11[c]	68± 4	1.4±0.1	0.7±0.1[c]	0.9±0.2
22	9	4.62±0.25[c]	91.1± 7.8[c]	165±24[c]	85±11	1.8±0.2[c]	0.9±0.1[c]	0.2±0.1[c]

[a] The data for Table 1 were taken from Daston and Grabowski (1979) and Daston (1982).

[b] Values are reported as mean ± SE. GD 22 Cd-treated are statistically compared to GD 21 controls.

[c] Significantly different from controls by Student's t-test ($p<0.05$).

The surge in pulmonary surfactant PC accumulation at the end of gestation is caused by an increased activity of the PC-synthesizing enzymes. Three of these, cholinephosphotransferase, lysoPC:lysoPC acyltransferase (Farrell et al. 1974; Oldenborg, van Golde 1976), and CTP:phosphocholine cytidylyltransferase (Weinhold et al. 1973) increase in activity at the end of gestation in the fetal rodent lung. Diminished activity of these enzymes could result in the slow rate of PC accumulation observed here.

Pulmonary Surfactant Synthesis

Synthesis rates for PC, DSPC, and SPH were determined in GD 20 and 21 control and GD 20 to 22 treated fetuses by measuring the amount of [^{14}C]choline incorporation. Data from this experiment are listed in Table 2. SPH synthesis more than doubled from GD 20 to term in both groups, and there were no differences between the groups. Total PC and DSPC synthesis rates also increased after GD 20. However, the increase in treated fetuses was approximately 25% lower than the controls. This was significant except for DSPC synthesis on GD 22, when there was a high standard deviation in the treated group. Even so, the difference was close to statistical significance ($p<0.10$). Thus, maternal Cd administration affects PC synthesis in the fetal lung. The half-life of PC in the airspace has been reported to be low--about 14 hours (Tierney et al. 1967). Thus, even though the prenatal PC concentration was adequate in the lungs of Cd-treated fetuses (Table 1), the lowered synthesis rate may render these rats susceptible to neonatal RDS.

The synthesis pathways of SPH and PC from choline share common steps (reviewed in van Golde 1976). Because the synthesis rate of SPH was unaffected by the Cd treatment, the synthetic steps for this phospholipid were probably not altered. Thus, a decrease in PC synthesis must result from an alteration in the anabolic pathway beyond the steps in common with SPH synthesis. Since the next step after the common pathways results in the synthesis of PC, cholinephosphotransferase, the enzyme responsible for this step, may have decreased activity in the lung of the term Cd-treated fetus. Cholinephosphotransferase activity increases sharply at the end of gestation in the fetal rodent lung (Farrell et al. 1974; Oldenborg, van Golde 1976)

Table 2. Effects of 8 mg/kg/day $CdCl_2$ on Pulmonary Surfactant Synthesis[a]

Treatment and Gestation Day	Number of Litters	Incorporation of $[Me-^{14}C]$Choline (dpm/mg tissue)		
		Total PC	DSPC	SPH
0 mg/kg				
20	10	39 ± 5[b]	12 ± 2	0.9 ± 0.2
21	10	81 ± 7	18 ± 2	2.0 ± 0.3
8 mg/kg				
20	10	33 ± 3	10 ± 1	0.7 ± 0.1
21	10	61 ± 7[c]	13 ± 1[c]	1.6 ± 0.3
22	10	55 ± 5[c]	14 ± 3	2.4 ± 0.5

[a] The data for Table 2 were taken from Daston (1982).
[b] Values are reported as mean ± SE. GD 22 Cd-treated are statistically compared with GD 21 controls.
[c] Significantly different from controls by ANOVA, $p<0.05$.

and this surge in activity might be blocked by the maternal Cd exposure.

Developmental Pattern for Pulmonary Glycogen

Another explanation for the decreased PC synthesis may be inadequate concentrations of precursors. Carbohydrates probably contribute substantially to pulmonary surfactant phospholipid synthesis, and a large carbohydrate deposit, as glycogen, is present in the fetal rat lung (Maniscalco *et al*. 1978). I measured the developmental pattern of pulmonary glycogen in Cd-treated and control fetuses (Table 1). Glycogen accumulates in control lungs until GD 20, after which it is apparently catabolized and falls to extremely low levels. The decrease in pulmonary glycogen content occurs at the same time as the surge in PC accumulation. The pulmonary glycogen concentration of the

Cd-treated fetuses was significantly decreased throughout late gestation. The diminished lung glycogen concentration may result in a decreased availability of glycerol-3-phosphate, a necessary precursor for PC synthesis. Hence, a Cd-induced decrease in the fetal lung glycogen content may result in decreased synthesis of surfactant PC. Synthesis of SPH would not be affected because this phospholipid does not contain glycerol.

Ultrastructural Observations

The histogenesis of the alveoli was observed to assess whether the alterations in biochemical development could be correlated with changes in microanatomical development. Normal cytodifferentiation of rat alveolar epithelium has been previously described (Hitchcock O'Hare, Sheridan 1970; Williams 1977). Normal differentiation of alveolar epithelial cells first becomes visible on GD 19 in the rat. Before this, the alveoli are lined by an undifferentiated epithelium of columnar cells with extensive apical and basal glycogen deposits. On GD 19, type II cells first become apparent with the formation of the surfactant-containing lamellar bodies. Cytodifferentiation continues with the continued formation of lamellar bodies, the thinning of the columnar epithelium to a cuboidal (type II cell) or squamous (type I cell) shape, and the disappearance of glycogen on GD 21. Quantitative data on the lamellar bodies and type II cells are presented in Table 3.

Differentiation of the alveolar epithelium in Cd-treated fetuses began, for the most part, at least a day later than in controls. By GD 21, many alveoli from treated fetuses appeared to be normally differentiated; however, the development of epithelium in many alveoli was delayed, and some possessed only the undifferentiated cell type observed before GD 19 in controls. By GD 22, more differentiation had occurred in the lungs of the Cd-treated fetuses, especially in the type I cells, but these fetuses still were deficient in type II cells and lamellar body number as compared with term controls. The effect on type II cells may not be real, since the sampling method did not include serial sections of the same cell, and a cell with few lamellar bodies, not in the plane of the section, would not be scored as a type II cell. On the other hand, the reduction in the number of lamellar bodies is credible, as

Table 3. Effects of 8 mg/kg/day $CdCl_2$ on Alveolar Histogenesis[a]

Treatment and Gestation Day	Number of Individuals Scored	Number of Type II Cells Per Ten Alveolar Lining Cells	Number of Lamellar Bodies Per Ten Alveolar Lining Cells
0 mg/kg			
19	3	1.1 ± 0.1[b]	1.3 ± 0.3
20	3	3.1 ± 0.9	7.2 ± 2.2
21	3	3.6 ± 0.4	11.7 ± 1.3
8 mg/kg			
19	4	0.4 ± 0.3[c]	0.4 ± 0.3[c]
20	3	0.9 ± 0.2[c]	1.2 ± 0.4[c]
21	4	1.9 ± 0.7[c]	3.6 ± 1.8[c]
22	7	2.2 ± 0.3[c]	5.6 ± 1.1[c]

[a] The data for Table 3 were taken from Daston (1981a).

[b] Values are reported as mean ± SE. GD 22 Cd-treated are statistically compared with GD 21 controls.

[c] Significantly different from controls by Student's t-test, $p<0.05$.

this correlates well with the reduction in pulmonary surfactant accumulation.

A delay or even an arrest in the development of some alveoli was observed in the lungs of Cd-treated fetuses. A decreased number of surfactant-containing lamellar bodies and diminished cellular glycogen deposits were also noted. This change in microanatomy correlates well with the data on biochemical maturation of the pulmonary surfactant and pulmonary glycogen reported above.

Postnatal Observations

A number of Cd-treated and control dams were allowed to give birth, and their offspring were observed for signs of respiratory dysfunction. Parturition was delayed for approximately 24 hours in Cd-treated dams, and litter size and birth weight of the offspring were decreased. None of the control offspring observed showed any respiratory problems. Six Cd-treated neonates (11% of the total number observed) developed respiratory problems beginning about 6 hours after birth. These problems included cyanosis and dyspnea. One of the neonates recovered 6 hours after the onset of the symptoms. The others succumbed to the respiratory distress. Upon autopsy, the lungs of these neonates were purplish-red, a sign of RDS/hyaline membrane disease in humans. Extensive atelactasis and refractile eosinophilic membranes in the airspaces were visible on histological examination (Daston, Grabowski 1979).

Dose-Response Study

I demonstrated that prenatal exposure to an environmental agent can alter biochemical parameters in the developing lung, including the synthesis and accumulation of the pulmonary surfactant. This is correlated with alterations in ultrastructural development, and may result in a postnatal functional deficit that resembles human neonatal RDS. Unfortunately, this was accompanied by a great deal of fetotoxicity, including a decrease in fetal lung weight, and therefore, it was unclear whether a functional deficit was any more sensitive than a morphological anomaly as an indicator of developmental toxicity. To determine whether the deficit in pulmonary surfactant maturation would persist in the absence of any other effects, we used progressively lower maternal doses of $CdCl_2$--0, 1, 2, 4, or 8 mg/kg $CdCl_2$ daily on GD 12 to 15. A dose-related decrease in litter size, fetal weight, lung weight, and pulmonary PC was observed on GD 21 (Table 4). A maternal dose of 1 mg/kg caused no alterations from controls in any of the morphological parameters; however, fetal pulmonary PC content was still significantly decreased in this dosage group. This demonstrates that the functional deficit persists in the absence of overtly detectable fetotoxicity and that deficits in function may be the most sensitive indicators of developmental toxicity.

Table 4. Effects of Lower Doses of $CdCl_2$ on Fetal Lung Parameters on GD 21

Dose (mg/kg)	Number of Litters	Fetal Weight[b] (g)	Lung Weight[b] (mg)	Lung PC[b] (μg)	Lung PC/Lung Weight (μg/mg)
0	11	5.48 ± 0.20[c]	163.8 ± 4.8	214 ± 16	1.3 ± 0.1
1	5	5.48 ± 0.22	162.7 ± 8.5	157 ± 22[d]	1.0 ± 0.1[d]
2	9	4.85 ± 0.16[d]	138.0 ± 6.1[d]	168 ± 12[d]	1.2 ± 0.1
4	6	4.40 ± 0.43[d]	118.0 ± 17.5[d]	128 ± 30[d]	1.2 ± 0.1
8	8	4.14 ± 0.23[d]	102.6 ± 10.6[d]	137 ± 11[d]	1.4 ± 0.1

[a] The data for Table 4 were taken from Daston (1981b).

[b] Significant dose-related response for the parameter, ($p<0.05$).

[c] Values reported are mean ± SE.

[d] Significantly different from controls by Student's t-test, ($p<0.05$).

CONCLUSIONS

Maternal exposure to 8 mg/kg $CdCl_2$ on GD 12-15 altered the pattern of PC accumulation in the fetal lung, which resulted in lowered absolute quantities of PC, the principal component of pulmonary surfactant. Although the concentration of pulmonary PC in the term Cd-treated fetus was not decreased, the rate of synthesis was decreased. The lowered rate of synthesis along with the relatively brief half-life of PC in the respiring lung may predispose these animals to respiratory difficulties postnatally. Difficulties in respiratory function were observed postnatally, with symptoms resembling human neonatal RDS/hyaline membrane disease. The 8 mg/kg/day dose produced embryolethality and gross morphological alterations along with the functional deficits; however, the decrease in fetal lung PC accumulation persisted even at a dose of 1 mg/kg/day, which produced no other observable signs of developmental toxicity. Thus, the alteration in the biochemical maturation of pulmonary surfactant appears to be the most sensitive indicator of toxicity. Alterations in the microanatomical development of the alveoli were also noted, and many of the ultrastructural observations correlate well with alterations in biochemical development. Hence, studies on ultrastructural development may serve as a useful adjunct to studies on functional development.

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DISCUSSION SUMMARIES--LUNGS

NORMAL AND ABNORMAL STRUCTURAL DEVELOPMENT OF THE HUMAN LUNG
(Claire Langston)

DR. KAVLOCK: Is the kidney/pulmonary proline connection unique to humans?

DR. LANGSTON: I think it occurs in a lot of species, but I'm not sure how many.

DR. ROBERTSON: Amniocentesis is the simple insertion of a needle into the amniotic sac with the removal of a very small amount of fluid. How can you account for the temporary removal of a tiny amount of fluid causing lung hypoplasia?

DR. LANGSTON: I did not say pulmonary hypoplasia. The Working Party on Amniocentesis did document two problems: an increased number of infants were born who had unexplained respiratory problems, and there was an increase in postural malformations. They also showed the other known associations of amniocentesis. The study on monkeys is a preliminary report from Allison Hislop's lab, and there's not much data available, but I think what's present is quite reliable. They did amniocentesis at two times in monkeys, one approximating 17 to about 20 weeks gestation and the other 22 to 25 weeks gestation in human terms. The early amniocentesis group had both depressed birth weight and lung weight when compared with controls. There were structural changes in the lung in both groups. The implications of this are unknown, but should be seriously considered.

DR. McCRORY: I'm intrigued by your comment about proline and hydroxyproline being made by the kidney. Were you speaking of its presence in amniotic fluid by fetal urine, or is there actual production?

DR. LANGSTON: An arginase in the fetal kidney produces most of the proline that's present early in embryonic development.

DR. McCRORY: You mentioned that hypoplasia of the lung was partly the result of space-occupying lesions. What is the result after removal of the space-occupying lesion in functional development of the lung?

DR. LANGSTON: Several studies partially address this problem. The lungs of the babies who survive do grow, but the structural development is not normal.

THE REGULATION OF FETAL LUNG DEVELOPMENT (Ian Gross)

DR. BARTOLOME: Have you done similar studies rendering the animals hypothyroid?

DR. GROSS: No, but other people have, and their studies suggest that hypothyroid animals do have delayed lung maturation. There's also evidence that human infants with hyaline membrane disease have lower thyroid indices. I'm not sure if there is a causal relationship.

DR. CROCKER: Is dexamethasone a naturally occurring compound?

DR. GROSS: No, but corticosterone and cortisol compete for the same receptors as dexamethasone, although with different affinities. The natural corticosteroid in the rat is corticosterone. However, dexamethasone is convenient to use for assays of steroid binding for a number of reasons: one is that dexamethasone is not a naturally occurring steroid, and another is that it resists metabolism.

DR. CROCKER: In the kidney dexamethasone seems to have a different pattern of binding than the naturally occurring steroids. Is the situation with dexamethasone so artificial that what you're now seeing never occurs in nature?

DR. BALLARD: The kidney has at least three types of binding sites: the mineralocorticoid, the glucocorticoid, and the corticosterone sites. The situation seems simpler in the lung because only one class of sites has been identified, and they bind both the dexamethasone and the natural corticoids.

HORMONES AND RECEPTORS IN DEVELOPING LUNG
(Philip L. Ballard)

DR. CROCKER: I understand that steroid action is cell-cycle-phase specific and has its major effect early in the cell cycle. And yet the receptor numbers increase independent of the effect of the steroids. Is my interpretation correct?

DR. BALLARD: In the models that have been examined, receptor numbers varied during the cell cycle.

DR. SLOTKIN: You have to consider the controversy. You may have more receptors, but you have to know what proportion of them are coupled to responsive units in the tissue. So the number of receptors alone doesn't tell you the biological function.

DR. BALLARD: In terms of glucocorticoid receptors, there seems to be a one-to-one relationship between occupancy of sites and response, at least in our organ culture system. So the more receptors you have, the more response you get.

DR. GARY KIMMEL: Whether it is T3 or even steroids, the steroids appear to function maximally even at levels well below maximum binding. There does seem to be in certain situations a plethora of binding sites, certainly more than are necessary to stimulate a particular maximum.

DR. BALLARD: That's particularly true of the membrane receptors where spare receptors exist. Glucocorticoid and thyroid hormone do not have spare receptors, so you get a one-to-one correlation between the number of sites occupied and the response, at least in those limited systems that have been examined.

DR. CROCKER: It doesn't occur in all the phases of the cell cycle; is that correct?

DR. BALLARD: I agree with you, but the cells are not synchronized. If you take a whole population of cells, they're going to be in different parts of the cycle at any given time.

DR. GROSS: Late in gestation the number of steroid receptors increases. Toward the end of gestation there may be little or no response to corticosteroids in terms of this particular mix, but that's because that system has already matured and is no longer inducible. So, the steroid has done its work. Early in gestation they may have a more marked effect because that maturation process has not been induced.

DR. SPITZER: You have shown some data on changes in receptor affinity for T3. I don't remember seeing anything similar for glucocorticoids. Is that true?

DR. BALLARD: Actually, the K_D for T3 binding is not statistically different in the fetal and the adult rabbit. In glucocorticoid binding, we find no difference in receptor affinity with age, but receptor capacity does increase during fetal life.

DR. SPITZER: And after birth does it decrease or stay the same?

DR. BALLARD: It gets more complicated after birth because of the endogenous adrenal steroids. You have to adrenalectomize the animal to accurately assay receptor affinity. Most likely there are no major changes after birth.

DR. SLOTKIN: The β-receptor is one that is notoriously a spare receptor system, and based on your dihydroalprenolol binding in the β-methazone treatment where you've got an increase in binding, you said that these cells should be more responsive to catecholamines. Have you actually tested their response?

DR. BALLARD: No. We are going to explore this in organ culture measuring both the increase in receptor number and generation of cyclic AMP.

EFFECTS OF PRENATAL NITROFEN EXPOSURE ON POSTNATAL LUNG FUNCTION IN THE RAT (James A. Raub *et al*.)

DR. LANGSTON: What do the lungs look like?

MR. RAUB: Histopathology in the lung was negative at both three and six weeks. So here we have a normal histopathological condition with the abnormal functional association.

DR. SPITZER: You said it might interfere with collagenesis. Did you do any special staining to see if collagen is there?

MR. RAUB: We did not do it in this study. We were more interested in the functional changes than in a structural basis for it. That's why I hesitate to call this a true case of hypoplasia: we may have structural abnormalities of the lung (i.e. in collagen or something) that weren't discernible by light microscopy.

DR. SPITZER: What is the evidence that this interferes with collagenesis?

MR. RAUB: This was presented in a paper by Stone and Manson, 1981, as a possibility based on the previous indications of fibrosis.

DR. CROCKER: Did you analyze for a chemical that you recovered from your model, or is it a metabolite, or does it work another way?

DR. GRAY: Drs. Manson and Costlow in Cincinnati have just published a paper on its metabolism that seems to indicate that it's the parent compound.

MR. RAUB: Also, the half-life is very short in the embryonic compartment, about 24 hours. In maternal blood the half-life is about 8 days.

DR. KAVLOCK: I'd just like to add that lung weights in the female offspring were affected to a greater degree.

MR. RAUB: The animals presenting with diaphragmatic hernias usually die of respiratory distress. At three weeks of age, only two of the animals we looked at had diaphragmatic hernias on the left dorsal section and survived to go into our evaluation.

DR. LANGSTON: Did you exclude those?

MR. RAUB: They were involved only in the histopathology evaluation.

PARACRINE REGULATION OF LUNG GROWTH AND MATURATION: THE SUBSTRATE OF NORMAL FUNCTIONAL DEVELOPMENT (Barry T. Smith _et_ _al_.)

DR. McCRORY: Before we looked for somatomedins, our hypothesis was that the organ has to expand by blood flow, hemodynamics, etc., and that would be the trigger for growth. That wasn't true. A factor was present in serum very shortly that was not there in 5, 6, and 7 days. Could timing have something to do with your failing to find in blood somatomedins when present in the tissue?

DR. SMITH: I'm fairly comfortable with that fact that somatomedins, at least, aren't appearing in serum. On the other hand, other data suggest that other factors are produced and circulated, and one from the kidney may in fact cross-react with the lung, whereas the one from the lung may not cross-react with the kidney. So I think, again, we're only probably looking at a very thin slice of the total phenomenon. Many details need to be worked out, and there could be two factors working, probably more. I assume, too, that they might be working in parallel or in sequence and it's going to take a lot of work to resolve this situation.

EFFECTS OF MATERNAL CADMIUM EXPOSURE IN THE RAT ON PRENATAL MATURATION OF THE PULMONARY SURFACTANT SYSTEM

(George P. Daston)

DR. SLOTKIN: I'm rather struck by the big decrease in glycogen content that occurs within a very short span. Usually this happens in a tissue because there's been phosphorylase activation, and that occurs through a number of very limited means. I think we can rule out some of the most typical means because we know they don't occur near term, such as thyroid hormone surge, glucocorticoid surge, and sympathetic nerve stimulation. And that leaves insulin and adrenal catecholamines as likely possibilities. Is there any evidence of phosphorylase activation occurring in the tissue that could account for the drop in tissue glycogen, and is there any evidence for a surge in insulin or adrenal catecholamines near term that could account for that?

DR. DASTON: Those are very good questions for which I do not have answers.

DR. SLOTKIN: The reason I think that it might be a relevant question is that part of your problem with cadmium might not be a deficiency. You're only measuring at 24-hour intervals, and in each 24-hour interval, glycogen concentration goes from low to high to low. So you could have a 12-hour shift in that surge that's making phosphorylase change. To interpret that as a deficiency of glycogen might be a little dicey unless you can show what's happening in the embryo.

DR. GROSS: This is the only situation I know of where there's a falling glycogen associated with a phosphatidylcholine decrease. Whether phosphorylase is involved in the rat lung in decreasing glycogen is also questionable.

DR. SLOTKIN: Is something happening in that 12-hour period in which the level is spiking? You may be in a premature spike and fall.

DR. DASTON: One important point to consider is that cadmium doesn't get into the fetus. It is stopped by the placenta. If there is an effect, it's indirect. It is mainly in the maternal liver and in the maternal kidney.

DR. SPITZER: How about the fetal kidney?

DR. DASTON: Cadmium can't get across the chorioallantoic placenta, which in the rat forms around GD 12. Other people have shown a placental dysfunction specifically in zinc transport capabilities in the placenta. Zinc levels in the fetus are reduced after maternal cadmium administration. I believe that there's a fetal zinc deficiency caused by the maternal cadmium treatment. Maternal zinc deficiency has been shown to affect the lung and the pulmonary surfactant.

DR. GROSS: Did you measure blood glucose in these fetuses?

DR. DASTON: No.

DR. LANGSTON: I'd like to make two comments. Though glycogen is seen in great numbers in the rat, the mouse, and the puppy and probably in other experimental animals as well, it really isn't present in the human fetal lung in the type II epithelial cell in the last half of gestation. It's probably present very early, exactly when I'm not sure. Also, I really can't resist commenting about functional and structural changes. I suppose it's an axiom of pathology that there are no functional changes that do not have structural correlates. It's a matter of looking for the right thing in the right place; it will always be there.

DR. KAVLOCK: When a teratologist talks about structure versus function, it's not the same as a pathologist doing so.

CARDIOVASCULAR PROBLEMS OF THE NEWBORN AND THEIR ETIOLOGIES

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INTRODUCTION

Abnormal cardiovascular function is usually a consequence of structural change. Most of my efforts as a pediatric cardiologist are directed at trying to reverse the hemodynamic disorders that are direct results of malformations occurring during the development of the heart and great vessels, especially those that occur during the first trimester of pregnancy. In this paper, I review the clinical problems we encounter and then search in more depth for methods by which we can study functional abnormalities during fetal life. These methods involve measurement in our patient population and in large animals primarily, but may suggest applications also for investigations involving smaller animal models.

First, let us consider the clinical predicament of the neonatal cardiologist. We see infants who have survived gestation and who present often as emergencies with respiratory symptoms, cyanosis, or congestive failure. Historical information is usually fragmentary and often the mother is unavailable for questioning. With the need for rapid diagnosis and treatment, there is little time for probing for suspected etiologies or teratogens. In fact, we don't know how to take a history for exposure to toxins other than those actually ingested by or administered to the mother and we may or may not know all the drugs that the mother has taken. The obstetrician has made value judgments in selecting potentially dangerous drugs, using risk/benefit considerations, for a very large number of medications. We look for features of syndromes, concentrating on the infant's appearance, but we also consider the specific heart lesions that might help us identify any causative agents.

Table 1 lists some of the potential teratogens, the ranges of frequencies of the cardiovascular diseases encountered, and the most common malformations seen in the heart. Several important aspects relating to these syndromes will be discussed. The list is not complete, but it represents some conditions that are especially interesting because of their cardiac manifestations. The frequencies of cardiac malformations listed are merely estimates, but these do reveal a wide range. Also, there are considerable differences in the cardiac conditions that predominate in these syndromes. Even though the studies delineating the specific lesions involve small numbers, some of these distributions are very different from the spectra of cardiac lesions occurring when isolated lesions are tabulated.

After the heart has passed through an embryogenic period of vulnerability for a particular lesion, exposure to a teratogen cannot produce the lesion (Nora _et_ _al_. 1974). An example of this is the case of conotruncal defects, such as transpositions of the great arteries, and tetralogy of Fallot, where exposure after 34 d cannot produce these lesions. Thus, before establishing the teratogenicity of a particular agent in the human, attention must be paid to the particular types of cardiac defects present. The timing of these vulnerable periods in humans has been obtained from data from thalidomide exposures; this knowledge represents the only positive benefit from that disasterous epoch.

ALCOHOL

This enormously important syndrome, whose story is well known, illustrates the important role that clinicians have played in defining the clinical consequences of known teratogens. David Smith's perceptions, which emphasized the extremely deleterious effects of heavy alcohol ingestion in pregnancy, brought out a syndrome that was dormant for many thousands of years (Hanson _et_ _al_. 1976; Smith 1976). Smith has boosted the clinician's role in teratology by identifying this syndrome, encouraging the clinician to

Table 1. Potential Teratogens, Frequencies of Cardiovascular (CV) Diseases, and Common Malformations[a]

Potential Teratogen	Frequency of CV Disease	Most Common Malformations
Alcohol	25 - 30%	VSD, PDA, ASD
Amphetamines	?5 - 10%	VSD, PDA, ASD, TGA
Anticonvulsants		
Diphenylhydantoin	6%	PS, AS, Coarct., PDA, VSD
Trimethadione	15 - 30%	TGA, T/F Hypoplastic left heart
Lithium	10%	Ebstein's, tricuspid atresia, ASD
Propranolol	?	Bradycardia
Sex Hormones	2 - 4%	VSD, TGA, T/F
Thalidomide	5 - 10%	T/F, VSD, ASD, Truncus
Warfarin	?	Truncus
Rubella	35%	PPS, PDA, VSD, ASD
Diabetes	3 - 5% (30 - 50%)	TGA, VSD, Coarct (For cardiomyopathy)
Lupus	?	Heart block
Phenylketonuria	25 - 50%	T/F, VSD, ASD

[a]Adapted from Nora _et al_. (1981).

diagnose the deleterious exposure from the clinical features in infants. However, soon after birth the dysmorphic external features may not be obvious, and the cardiac findings themselves may lead us to suspect a particular syndrome. The distribution of cardiac lesions in the fetal alcohol syndrome is of interest on several counts. First, the lesions that predominate, atrial and ventricular septal defects, are not as severe as some of the lesions predominating in other syndromes. The earlier clinical impression was that ventricular septal defects predominated, but cardiac catheterizations have revealed that atrial septal defects are the major persistent lesions (Hanson *et al*. 1976; Loser, Majewski 1977).

AMPHETAMINES

One report of transposition from use of dextroamphetamine (Dexedrine®) during the first trimester has alerted us to ask about maternal use of this drug when transposition appears in the offspring (Nora *et al*. 1981).

DIPHENYLHYDANTOIN

The 6% risk of serious birth defects in women taking diphenylhydantoin (Dilantin®) during pregnancy is well established. Of the two cases we encountered during the last year, both infants had ventricular septal defects and the mothers were taking phenobarbital concurrently for seizures. Various other congenital heart lesions may also be present. The chief external diagnostic clue is the small nails often seen in these dysmorphic babies (Smith 1976).

TRIMETHADIONE (TRIDIONE®)

Tridione® has a high incidence of cardiac anomalies (see Table 1). This should be reason enough to avoid use of this drug during pregnancy, because the risk/benefit ratio is clearly excessive. Unlike diphenylhydantoin, trimethadione is not essential in the treatment of many cases of epilepsy (Smith 1976).

LITHIUM CARBONATE

An interesting relationship has been shown between mothers treated with lithium, usually for depression, and

Ebstein's anomaly of the heart (Nora et al. 1974). This anomaly involves displacement of the medial (septal) and posterior tricuspid valve cusps into the right ventricle away from the AV junction. This cardiac condition is quite unusual and the specific nature of lithium's role in producing the lesion is somewhat unique and deserving of additional study. The diagnosis of a recent case at the North Carolina Memorial Hospital was confirmed by echocardiography (Fig. 1) (Long, Willis 1983). Although Ebstein's anomaly is the most frequent consequence of lithium carbonate therapy, other conditions involving tricuspid valve dysfunction, including insufficiency and even atresia, have been reported (Nora et al. 1981).

SEX HORMONES

Estrogen and progesterone preparations, including contraceptives, have been considered as possible low-level teratogens (Heinonen et al. 1977; Levy et al. 1973; Nora, Nora 1973). This issue continues to be hotly disputed, however, and again reveals the dilemma of the obstetrician making decisions on the basis of risks and benefits. The massive use of these agents, estimated in 300,000 pregnancies yearly in the United States, could generate as many as 3,000 cases of congenital heart disease if this unproven relationship does exist (Nora et al. 1982).

DIABETES

The infant of the diabetic mother (Rowe et al. 1981) also deserves special mention, although the teratogenic agent has not been identified. The incidence of congenital cardiac manifestations is generally increased to a minor degree. Also, the well-described occurrence of asymmetric ventricular septal hypertrophy during fetal life in these infants may have special relevance to the etiology of idiopathic hypertrophic subaortic stenosis, an important disease in older children and adults. Fortunately, this condition recedes after several months of the infant's life. Its presence in an infant raises strong likelihood that the mother is diabetic or will be predisposed to developing diabetes.

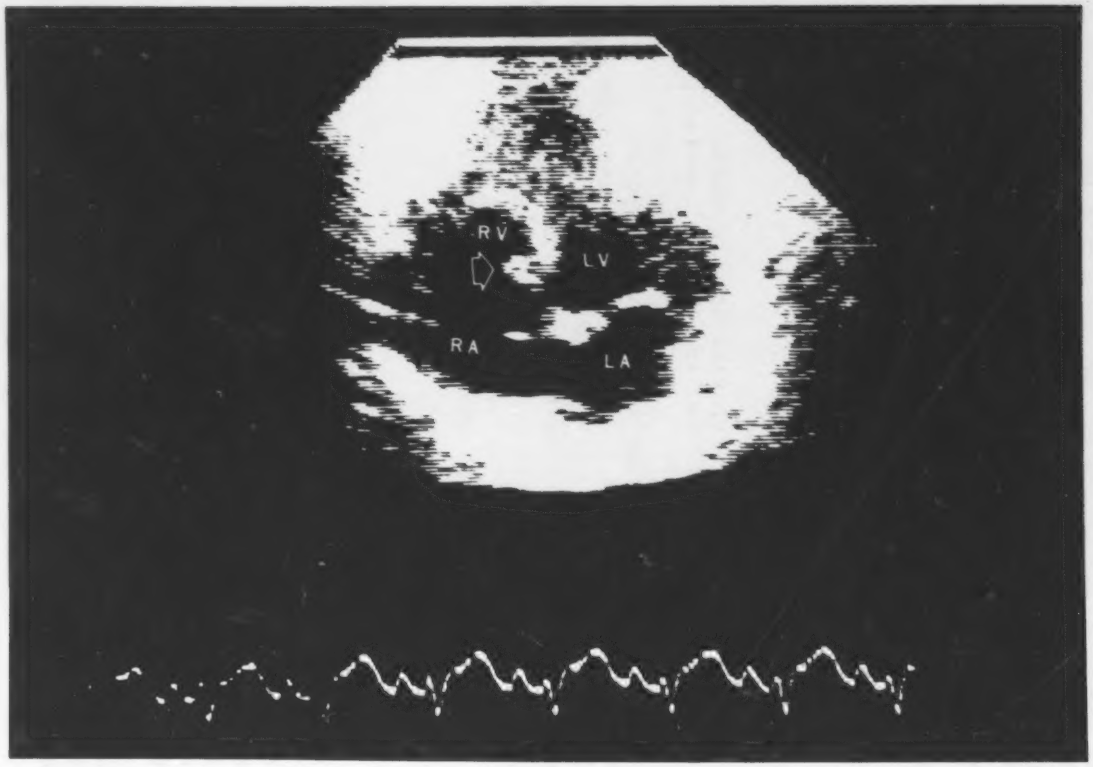

Fig. 1. 2-D echocardiogram revealing displacement of septal leaflet of tricuspid valve (see arrow).

Thus far, familial or genetic factors in the etiology of these syndromes or in the more straightforward cases of isolated cardiac defects have not been discussed. There is a wealth of information that genetic predisposition, primarily multifactorial, is usually involved in cases of isolated cardiac defects and that the deleterious environmental exposure has to occur at a time when the heart is undergoing specific embryologic changes (Nora *et al.* 1981). The specific mechanisms by which genes may affect detoxification of teratogenic substances are beginning to be revealed in one of the very exciting developments in this field (Kretchmer 1978).

Apart from the cardiac disorders involving malformations which develop in early pregnancy, there are a variety of conditions involving physiologic derangements which may develop long after the first trimester of pregnancy.

SALICYLATES

Salicylates are of particular interest in this regard and deserve more attention as possible deleterious agents during pregnancy than they have received. Salicylates are present in literally hundreds of medications as well as foods, and are difficult to monitor during pregnancy. They may prove to be human teratogens, cause excessive bleeding, and have late effects on the fetus through closure of the ductus arteriosus since this deleterious effect has been demonstrated in animals (Heymann, Rudolph 1976). In addition, salicylates have a profound effect on fetal breathing, causing a marked increase in its strength, which might not be related to ductal closure (Ballard 1983). This dynamic closure can be accomplished readily in the near term fetal animal (Heymann, Rudolph 1976). The role of prostaglandin synthetase inhibitors in permitting ductal manipulation of the newborn premature infant with respiratory distress has proven to be very important and accounts for a large number of consultations by cardiologists in the newborn nursery. In the last year, we treated over 50 such infants with intravenous lyophylized indomethacin, which has properties similar to aspirin in closing the ductus and which has a better reputation. Although this medical therapy is quite effective, it sometimes fails to close the ductus permanently, and surgery becomes necessary. About 15% of all cardiac operations on children at the North Carolina Memorial Hospital are done for this purpose.

NEONATAL CARDIAC CONDITIONS NOT ASSOCIATED WITH MALFORMATIONS

Apart from cardiac malformations, the most important cause of fetal cardiovascular dysfunction is asphyxia. Some of the major conditions associated with perinatal asphyxia are persistent pulmonary hypertension of the newborn ("persistent fetal circulation"); tricuspid valve apparatus dysfunction; hypoglycemia; hypocalcemia; and hypoxic damage to the myocardium and resultant congestive heart failure.

The persistent pulmonary hypertension syndrome has proven to be difficult to treat. In some cases, especially those where uterine asphyxia has been prolonged, efforts to overcome the persistently increased pulmonary vascular resistance fail completely. Hyperventilation of the infants to relieve hypoxia and hypercarbia, treatment with vasodilator agents, and general supportive measures are keystones to therapy.

Cellular functional derangements from hypoxia and acidosis result in the other major conditions. In cases of severe intrauterine asphyxia, these conditions are probably the immediate cause of death. In the survivors, attention to the infant's hypoxic state and to his acidosis, hypoglycemia, and hypocalcemia can often reverse even severely compromised cardiac output. The production of tricuspid valve regurgitation (Freymann, Kallfelz 1975; Bucciarelli *et al*. 1977), resulting in an identifiable systolic heart murmur, may indicate papillary muscle dysfunction and usually resolves in time. However, several autopsied patients have shown necrosis of the anterior papillary muscle of the tricuspid valve (Bucciarelli *et al*. 1977).

The focus of fetal monitoring is the identification of fetal hypoxia, but one can readily see that the monitoring methods can be adapted for monitoring large animals in experiments in which specific toxins are being studied. Table 2 lists the variety of monitoring techniques being used in humans.

Table 2. **Human Fetal Physiological Monitoring**

Auscultation for cardiac rate and rhythm
Echosonography: early for fetal size, position, placental location, major anomalies; later for specific cardiac diagnosis
Phonocardiography (non-invasive)
Fetal ECG
Relationships between heart rate and uterine contraction (dilated cervix with patient in labor)
Fetal-scalp monitoring

It is desirable to avoid invasive techniques. Detailed fetal ECG, contraction-heart-rate relationships, and blood-scalp monitoring need to be performed by direct invasion of the uterus. External ECG monitoring often does not provide adequate tracings of P wave morphology.

Creasy and Parer (1977) present the technique that is widely used for determining the effects of uterine contractions on heart rate (Fig. 2). From the types of cardiac decelerations produced, valuable information is obtained about the nature and severity of fetal distress. The basic obstetrical methods, which are well established, may have some applications for the experimentalist, and they are used to identify a variety of functional and structural disorders (Table 3)

Fetal cardiac functional studies present some technical problems, but they are possible. Heart rate and rhythm, LV shortening fraction, RV and LV systolic time intervals (with the accompanying ECG) can be determined.

The extraordinary non-invasive methods that are possible with echocardiography (Kleinman *et al*. 1982; Kleinman *et al*. 1980) should allow repeated observations of cardiotoxic effects of agents in large animals without

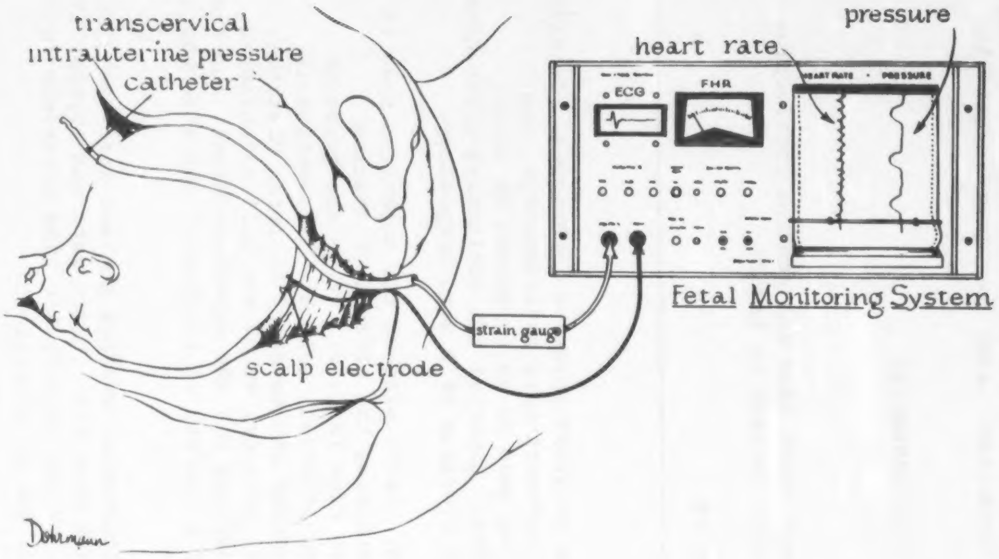
transcervical
intrauterine pressure
catheter
scalp electrode
strain gauge
heart rate
pressure
ECG
FHR
HEART RATE · PRESSURE
Fetal Monitoring System
Dohrmann

Fig. 2. Schematic representation of components for monitoring fetal heart rate and uterine contractions during labor.

Table 3. Cardiovascular Disorders Readily Diagnosed
in the Fetus

Tachycardia, bradycardia indicative of fetal distress

Rhythm disturbances, PAT, heart blocks, PVCs

Specific cardiac defect (by echo, especially 2-D)
- Tetralogy of Fallot
- AV Canal, ostium primum
- Hypoplastic right heart
- Hypoplastic left heart
- Cardiomyopathy
- Ebstein's anomaly
- Double outlet right ventricle

Congestive failure (hepatomegaly, ascites, edema)

Abnormal fetal breathing patterns

Abnormal cardiac function studies

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needing to sacrifice the animals. I am not aware of the scaling down of these instruments for use with fetuses that are smaller than human (or sheep) fetuses, but I believe that this should prove very useful.

OBSERVATIONS ON LARGE ANIMALS

Proceeding now from human observations to those in large animals, attention should be directed particularly toward the sheep model developed by Barcroft and Dawes using exteriorized lambs (Barcroft 1947; Dawes 1968; Harned *et al*. 1961) and perfected by Rudolph and others as a chronic fetal preparation with the mother on the hoof. Many of their methods are limited to larger animals, but these preparations lend themselves to repeated or serial observations providing much data. A partial listing of procedures that can be performed on these fetal, relatively undisturbed animals is shown in Table 4.

Table 4. Physiological Measurements in Sheep

Blood Sampling - Umbilical vein, umbilical artery, IVC, SVC, hepatic vein, ductus venosus, RA, LA, RV, PA, LV, proximal aorta, ductus arteriosus, distal aorta, coronary sinus, uterine artery and vein.

Electromagnetic flowmeter installation for flow readings of umbilical vein (single vessel in abdomen), umbilicial artery (arises as a single segment), IVC, SVC, PA, proximal aorta, distal aorta, ductus arteriosus.

Regional blood flows by microsphere method.

The array of measurements that can be performed indicates the versatility of the large animal preparation for observations during the latter part of pregnancy. As the prototype animal, the fetal lamb has been studied extensively and a great deal is known about its normal circulation. Some of the drugs that have been studied in sheep are listed in Table 5.

I cannot review the major drug studies that have been performed on sheep, but I would like to emphasize two particularly elegant techniques which might be applicable to studies of the pathophysiological effects of toxins on fetal circulation.

First, and basic to many studies by Rudolph and associates, is the use of radionuclide-labeled particles to determine changing blood-flow patterns in the fetus. The methods used are complex, and it requires considerable effort to establish them in a laboratory. Once established, however, a great deal of information can be gathered (Heymann et al. 1977).

Second, a very clever preparation has been set up for measuring cardiac metabolism. Fisher et al. (1980, 1982) took advantage of the fact that the left hemiazygous vein of the sheep drains into the coronary sinus. Insertion of a catheter 0.8 to 1.4 cm into this vessel and occluding it proximally ensures access to the coronary sinus blood

Table 5. Drugs and Drug Effects Studied in Sheep

Indomethacin, aspirin, naproxan
Ritodrine
Digoxin
Naloxone
Glucosteroids and B blockade
PA vasodilators
Angiotensin II
Hypoxia
Lidocaine

draining the left ventricular free wall. A left thoracotomy is necessary for this insertion, so the technique is limited to large fetuses. From appropriate AV-difference studies and later myocardial blood flow determinations by radionuclide microspheres, a variety of measurements of myocardial O_2 consumption, glucose, and lactate and pyruvate utilization, with comparisions with the adult or stressed animals, can be made.

The various rhythm disturbances encountered in the fetus and neonatal infant are interesting, but serious arrhythmias are quite unusual in a neonatal practice. They may be over-diagnosed, with inappropriate termination of pregnancy, especially since obstetricians consider fetal heart rates below 80 and above 210 "arrhythmias." In one series, the most frequent significant arrhythmia was premature beats (32%), followed by paroxysmal superventricular tachycardia (19%) and atrial flutter (16%) (Kallfelz 1979). A high degree of AV block, a condition that is easy to diagnose, was found in 24% of these cases. Repetitive tachycardia was found in 6% of the cases; ventricular tachycardia and atrial fibrillation occurred in only 0.8% of cases. I suspect that the incidence of ventricular tachycardia in this series is an underestimation.

Maternal anxiety, presumably resulting in release of catecholamines, has been associated with premature beats, which are said to be relieved by maternal sedation

(Bernstine et al. 1968). Potential cardiotoxic agents, such as digoxin, can cross the placenta and invoke arrhythmias. The most interesting etiology for arrhythmias is the strong association of maternal connective tissue disease (including disseminated lupus erythematosis) with various degrees of heart block, including complete A-V dissociation (McCue et al. 1977). The structural cause for this dissociation is lack of AV node formation or the encasement of the AV node in connective tissue. The fetus may develop a high-degree block when its mother has not shown flagrant signs of lupus and certainly has no conduction disturbance.

I know of no studies of possible damaging effects on the fetal conduction system from maternal ingestion of drugs, such as hydralazine, that may cause syndromes resembling lupus. With present diagnostic methods, particularly echocardiography, the various types of heart block can be diagnosed readily in the human fetus.

Fetal heart block with congestive failure, as diagnosed by echo, is cause for cesarian section and consideration of pacemaker insertion. Fetal tachyarrhythmias with failure can be treated with maternal digoxin treatment (reaching the fetus by placental transfer), by maternal propranolol (Teuscher et al. 1978), or by maternal procainamide therapy (Dumesic et al. 1982). In addition to the ability to diagnose these arrhythmias in utero, the echocardiogram permits differentiation of premature atrial from premature ventricular beats and identification of atrial flutter (Kleinman et al. 1982; Kleinman et al. 1980).

CONCLUSIONS

We are still very naive in our understanding of the effects of various deleterious agents on the vulnerable fetus and the mechanisms of the effects. Areas that I believe should have high clinical priorities include

1) Vigorous efforts to understand the mechanisms of fetal cardiovascular disorders from drugs abused by the mother, including alcohol. Physicians cannot control these agents.

2) Continued attention to problems related to some of the common agents that threaten infants, such

as salicylates and sex hormones and the effects of maternal smoking.

3) Development of techniques for improving history taking, especially as this applies to exposure to environmental agents such as pesticides and household toxic substances.

4) Improving the interchange of information between clinicians and experimenters both in conferences and in our respective journals. As I prepared this paper, I became increasingly aware of my need to know much more about the etiologies of the conditions with cardiac manifestations which are so devastating and very costly to treat. I believe external environmental agents will also be shown to cause some of these diseases.

Because almost all of the disorders that have been discussed in this article were discovered in the brief period since the thalidomide disaster, one can be sure that there are conditions caused by various agents that can be discovered by perceptive persons. The importance of external environmental agents in causing human cardiac malformations during embryogenesis or in causing fetal cardiac disorders developing later in pregnancy, or even neonatally, remains to be defined.

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THE ELECTROCARDIOGRAM OF FETAL AND NEWBORN RATS AND DYSRHYTHMIAS INDUCED BY TOXIC EXPOSURE

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INTRODUCTION

Cardiac dysrhythmias are not uncommon in human newborns. An incidence of 6% first-degree and 1.2% second-degree heart blocks was found in a group of neonates admitted to the newborn special care nursery of a large county hospital (Ferrer 1977). A recent request for research grant applications from the National Heart, Lung and Blood Institute of the National Institutes of Health (NIH) stated that about 2% of randomly selected children had some form of cardiac dysrhythmia, and that the etiology of acquired or naturally occurring dysrhythmias in the developing heart is unknown (NIH 1983). For the last 5 years, I have used an electrocardiographic method to study the effects of mirex, an organochlorine pesticide, on rat fetuses and newborns. I have found that prenatal exposure to this substance can induce a variety of dysrhythmias. These can be induced in good frequency (up to 50%) and by very low doses. This seems to be a specific effect on the fetal heart, and not a reflection of general maternal toxicity. The period before birth is more sensitive to this type of toxic effect than the earlier period of organogenesis. These studies of mirex will be reviewed, along with some observations on other substances which indicate that the capacity to induce cardiac dysrhythmias is not unique to mirex.

The observations, along with those of Watkinson (1983), demonstrate that a variety of dysrhythmias can be induced by prenatal toxic exposure, and consequently imply that some human pediatric problems might be caused by similar exposures. They also provide an animal model for the study of the development of dysrhythmias, and demonstrate the usefulness and desirability of screening for functional abnormalities as indicators of developmental toxicity.

THE ELECTROCARDIOGRAM OF NORMAL RAT FETUSES AND NEWBORNS

The equipment we use consists of an electrophysiological amplifier and a rapid action recorder capable of running at several speeds. Most of the work was done with a Tektronix 122 preamplifier and a Brush 220 recorder; more recently, a Gould 2200S recorder/preamplifier has been used. The signals are amplified between 500 and 1000 times. The testing is done in a galvanic cage because of this high amplification and the possibility of interference from other electrical equipment. The electrodes are pure silver wires of between 0.007 and 0.010 in. in diameter (depending on the age of the individual). We normally place these in positions comparable to leads I, II, and III of human use. The ECGs used to illustrate this report are all from lead II. In the prenatal studies, the fetuses are exteriorized one at a time and tested while still attached to the mother (Fig. 1) (Grabowski, Payne 1980; Grabowski, Payne 1983a). In the postnatal studies, the newborns are tested as soon as possible after birth (usually within 5 min) (Fig. 2), tattooed, and retested for several days afterwards (Grabowski, Payne 1983b). Initially we tested unanesthetized pups, but found that testing an active, healthy 5-d pup can be very exasperating. Consequently, in more recent studies we have anesthetized them for 30 s with light ether sufficient to keep them still for 30 to 60 s. This treatment has no significant effects on either rate of heartbeat or weight gain (Table 1). This latter fact demonstrates that the small amounts of anesthetic used do not affect food consumption or maternal acceptance.

The electrocardiograms we obtain are of good quality and suitable for both quantitative and qualitative analysis (Fig. 3). Tracings are obtained at three speeds: 5, 25, and 100 or 125 mm/s. The highest speed is appreciably faster than that used with humans, but it is necessary in order to properly visualize the components of the ECG at the high speed with which these events occur in small hearts.

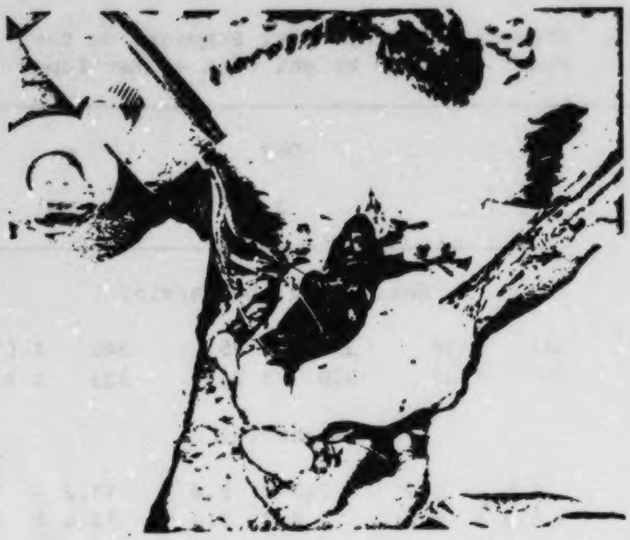

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Fig. 1. Setup for recording the ECG of an exteriorized rat fetus of 18.5 d. The electrodes are in the lead II position. The fetus is still attached to the mother via the umbilical cord and placenta.

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Fig. 2. An unanesthetized newborn having its ECG recorded.

Table 1. Effects of Light Ether Exposure on the Heart Rate and Weight Gain of Rat Pups[a]

	Day		
	1	2	3
		Heart Rate (beats/min)	
No Ether	320 ± 38	358 ± 25	340 ± 51
Ether	308 ± 39	328 ± 24[b]	339 ± 27
		Weight (g)	
No Ether	6.5 ± 0.7	7.7 ± 0.8	11.2 ± 1.3
Ether	6.3 ± 0.5	7.4 ± 0.4	12.6 ± 0.8

[a] Thirty to forty pups in each group.

[b] Significantly different, $P < 0.05$.

We have made extensive recordings of hearts of untreated rat fetuses and newborns at different ages to serve as standards. The rate of heartbeat changes rapidly throughout the fetal and postnatal periods (Fig. 4). Both PR interval (approximately a measure of conduction speed between atria and ventricles) (Fig. 5) and QT interval (a measure of recovery time of the ventricles) (Fig. 6) vary inversely with the rate of heartbeat.

Two benign types of arrhythmias were found in the control series at the late fetal and newborn stages. In one type, the one or two beats associated with the peak of a breathing effort were displaced, usually occurring somewhat prematurely. In the other type of arrhythmia, the rate of heartbeat would speed up or slow down for periods of 15 to 34 s. Both of these types occur in human newborns and are considered benign. These arrhythmias occurred in about 15% of the control animals (Grabowski, Payne 1983b) and always disappeared within 45 min of birth.

Significant abnormalities of the ECG were found in only 3 newborns out of the nearly 2000 fetuses and newborns that

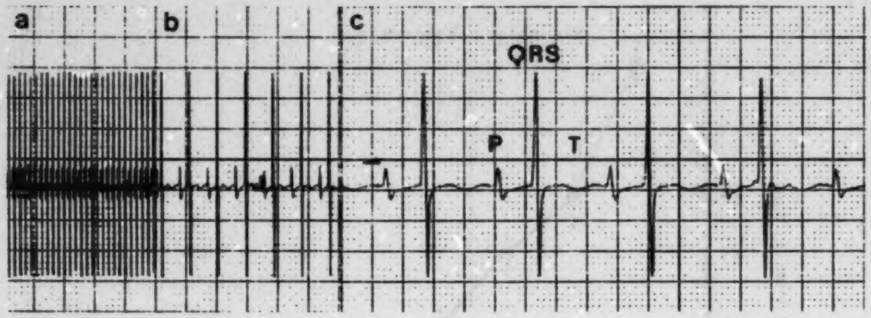

Fig. 3. An electrocardiogram from a 5-d-old rat pup (oil-fed control, lightly anesthetized with ether), taken at chart speeds of a) 5, b) 25, and c) 100 mm/s. The heart rate is 288/min. The P, QRS, and T components are labeled in 3c.

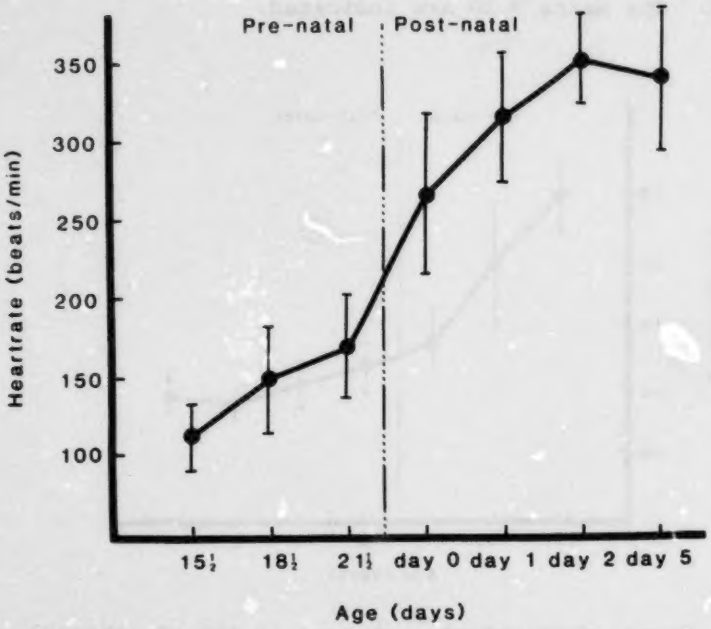

Fig. 4. Developmental changes in the heart rate of normal rat fetuses and newborns. The means ± SD are indicated.

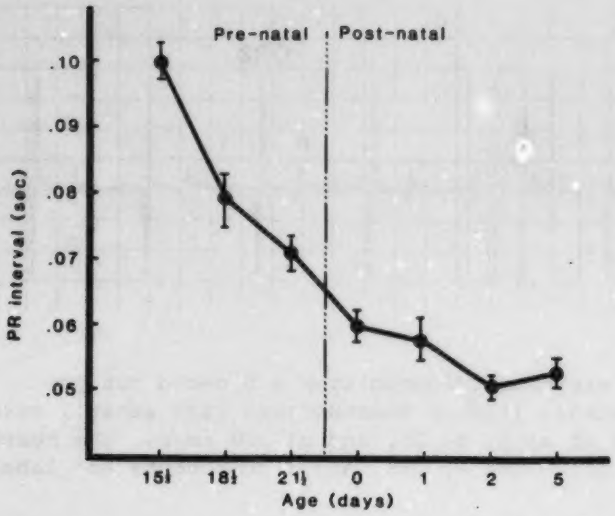

Fig. 5. Normal developmental changes in the PR interval, obtained from electrocardiograms of rat fetuses and newborns. The means ± SD are indicated.

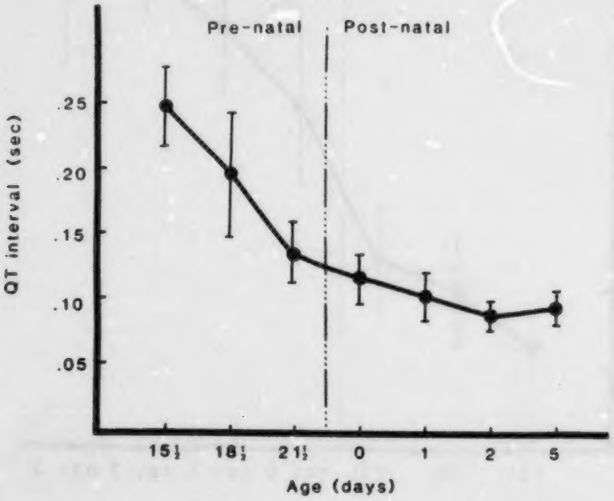

Fig. 6. Normal developmental changes in the QT interval, obtained from electrocardiograms of rat fetuses and newborns. The means ± SD are indicated.

we have tested to date. One of these was a transient period of premature atrial contractions. The other two individuals exhibited extreme bradycardia at birth and died within 30 min. They apparently died of noncardiac causes, and a slow heartbeat was only a reflection of other problems. Thus, the incidence of significant dysrhythmias in our control series is virtually zero.

EFFECTS OF MIREX ON THE FETAL ELECTROCARDIOGRAM

Mirex (dodecachlorooctahydro-1,3,4-metheno-1H-cyclobuta[cd]pentalene) is an organochlorine insecticide that was once widely used in the southeastern United States for the control of the fire ant (Solenopsis spp.). Its registration was canceled in the United States in June 1978, but efforts to have it reregistered are still active. The registration was canceled because of the toxicity of mirex and its very long half-life in the environment as well as in the body. The acute single-dose LD_{50} for adult female rats is 365 mg/kg; the 90-d dose LD_{50} is 6 mg/kg/d (Gaines, Kimbrough 1970). Signs of poisoning include diarrhea, weakness, and hyperexcitability, but the nature of mirex's toxicity is still very poorly understood (Waters et al. 1977).

I started to work on this substance because 1) it was known to induce a significant degree of fetal edema, and I was interested in the effects of fetal fluid imbalance; and 2) it was known to induce a high perinatal death rate (up to 50% after exposure to moderate doses) for which there was no explanation (Gaines, Kimbrough 1970; Khera et al. 1976; Chernoff et al. 1979a; Chernoff et al. 1979b; Grabowski 1981; Grabowski, Daston 1983; Grabowski, Payne 1980; Grabowski, Payne 1983a; Grabowski, Payne 1983b) because it induces few visible malformations.

In the first series of experiments, pregnant rats were fed from 5 to 10 mg mirex/kg/d during the period of organogenesis (Days 8.5 to 16.5), and the fetuses were examined 2 d after the last dose was given (Grabowski, Payne 1980). Many of the effects were proportional to the degree of visible edema, which was rated on a scale of 1 (slight swelling under the chin) to 5 (2.5 mm of subcutaneous edema visible across the back). Within the group exposed to 6 mg/kg/d (a moderate dose that caused no maternal deaths), the rate of heartbeat increased strikingly with increasing edema (Fig. 7). The most severely swollen fetuses, however, had a reduced heart rate, possibly an indication of heart failure. The PR intervals, normally inversely proportional to the heart rate (Figs. 4 and 5), increased with the heart rate in the ECGs of treated fetuses.

Prolongation of a PR interval beyond rather narrow limits in humans is a first-degree (1°) heart block. Since standards of this type do not exist for rats, we defined a 1° block for any age of fetuses or newborns as a PR interval greater than the mean of a comparable group of controls plus three standard deviations. By this criterion, from 20 to 77% of the treated fetuses had a 1° block (Fig. 8a) in dose-related incidence (Table 2). The ECGs of some treated fetuses showed missing QRS complexes, 2° heart blocks

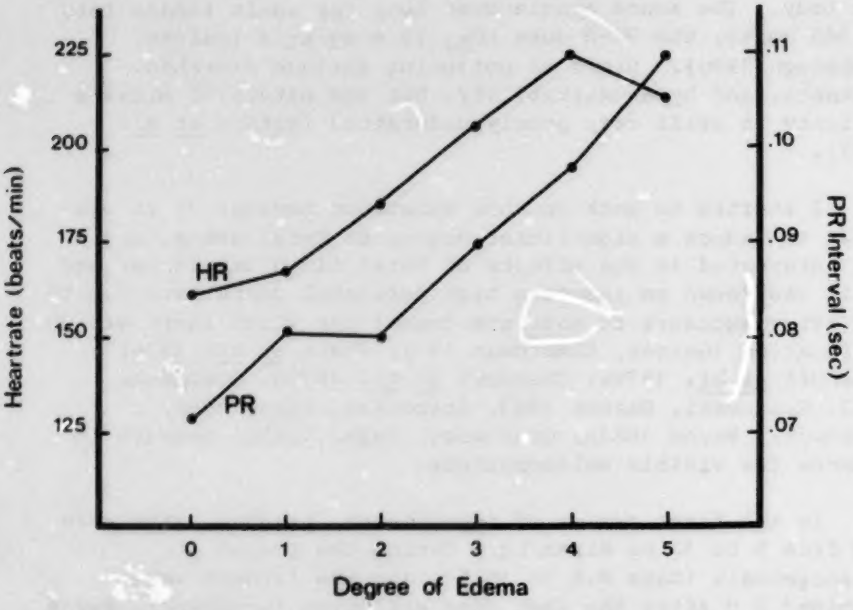

Fig. 7. The rate of heartbeat and PR intervals of mirex-exposed (6 mg/kg/d) fetuses, 18.5 d old, with different degrees of edema. [From Grabowski, Daston 1983. Used by permission.]

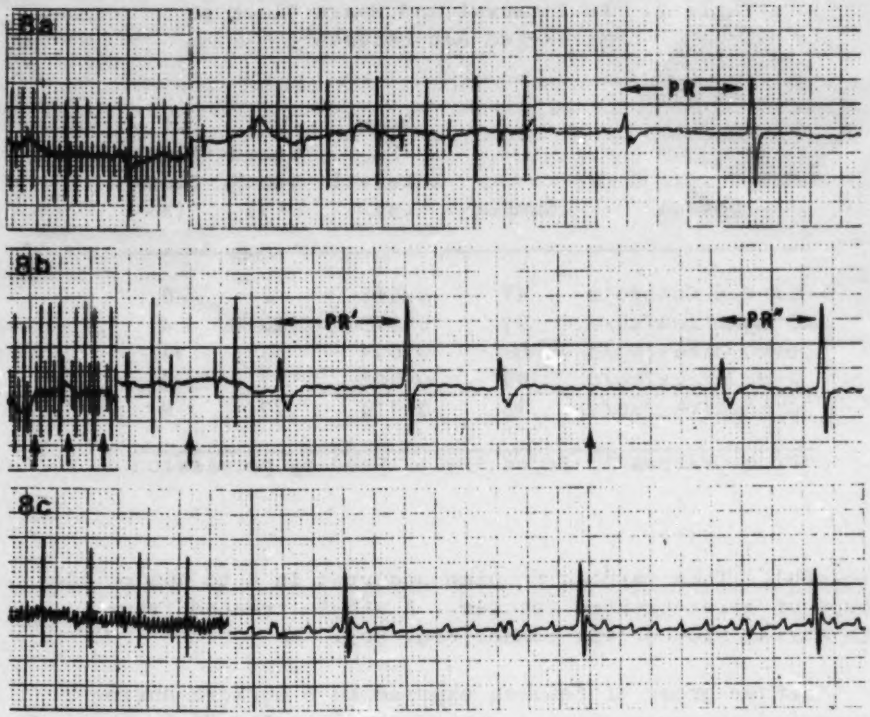

Fig. 8. Abnormal electrocardiograms obtained from mirex-treated (5 or 6 mg/kg/d) fetuses on Day 18.5 of gestation. A 1° heart block is shown in 8a. The PR interval is more than twice the average for this age, even though the heart rate (182/min) is somewhat above normal (150/min). A 2° heart block (Type I) is illustrated in 8b. The missing QRS complexes are indicated by arrows. The heart rate is 210/min; the PR intervals are prolonged and of variable duration. An ECG of a fetus with apparent atrial flutter is shown in 8c. The ventricles are beating at a rate of 204/min, the atria at 36/s. Segments of the record at 5, 25, and 125 mm/s are shown in 8a and 8b; only the 25- and 125-mm segments are shown in 8c. [From Grabowski, Payne 1980. Used by permission.]

Table 2. PR Interval and Heart Block in Mirex-Fed Rat Fetuses[a]

Dosage	Number	Mean PR Interval (s)	1° Heart Block (%)	2° Heart Block (%)
Oil-Fed Controls	47	0.065	0	0
5 mg mirex/kg/d	93	0.079	20	4
6 mg mirex/kg/d	60	0.091	50	10
7 mg mirex/kg/d	49	0.086	27	4
10 mg mirex/kg/d	13	0.110	77	8

[a]From Grabowski, Payne 1980. Used by permission.

(Fig. 8b). This cardiac problem occurred in 4 to 10% of the groups of mirex-treated fetuses. A single treated fetus with atrial flutter was found (Fig. 8c).

Another group of fetuses exposed to 6 mg/kg/d during the period of organogenesis was examined on Day 21.5, the morning before they were due to be born, to see if these symptoms persisted (Grabowski, Payne 1983a). The same cardiovascular problems were present and were now more severe. In addition to 1° and 2° blocks, dead and dying fetuses were evident, and acute bradycardia, inverted QRS complexes, and 3° blocks were found.

The effects of the moderate level of exposure (6 mg/kg/d on Days 8.5 to 16.5) on maternal hearts are currently being tested to see if the fetal effects are possibly a reflection of maternal cardiotoxicity. To date, neither dysrhythmias nor any other changes in the characteristics of the heartbeats in treated mothers have been found (Table 3). This is not surprising, since no cardiotoxic effects of mirex have been reported previously (Waters et al. 1977; Desaiah 1980).

This phase of the mirex study demonstrated that the treated fetuses exhibited a variety of cardiac problems: 1°, 2°, and 3° heart blocks; bradycardia; tachycardia;

Table 3. Effects of a Moderate Dose of Mirex (6 mg/kg/d) on the Hearts of Pregnant Rats at Day 20.5 of Gestation[a]

	Heart Rate (beats/min)	PR Interval (s)
Oil-Fed Controls (N = 5)	369 ± 87[a]	0.06 ± 0.01
Mirex-Treated (N = 8)	371 ± 79	0.06 ± 0.01

[a] Values shown are mean ± SD.

inverted QRS; atrial flutter; and possibly heart failure. Additional problems that were detected in the ECGs of mirex-treated fetuses of other series, mostly in low-dose studies, were premature atrial contractions, premature ventricular contractions, and atrial fibrillation. These dysrhythmias can occur in high frequency and seem to be fetus specific. Neither gross nor histological examination of these hearts has revealed any morphological basis for these functional problems.

THE EFFECTS OF PRENATAL EXPOSURE TO MIREX ON THE ECGs OF NEWBORN RATS

Electrocardiograms were obtained in newborn pups to see if the cardiovascular problems of mirex-exposed fetuses persisted after birth and contributed to postnatal death (Grabowski, Payne 1983b). A moderate dosage was used (6 mg/kg/d administered between Days 8.5 and 15.5). The first ECGs were usually obtained within 5 min of birth and subsequently at 1, 2, and 5 d of age.

Twenty-two percent of the pups were stillborn. Twenty percent of the pups were born with 1° heart blocks and 4% with 2° blocks. All of the latter group died within 30 to 60 min after birth; several of the former died within 24 h after birth. One exposed pup developed a long QT interval

(Fig. 9) on postnatal Day 1 and died several hours later. Respiratory problems also caused some deaths. However, it was clear that the dysrhythmias that originated before birth persisted into the postnatal period and appeared to be responsible for many of the deaths caused by mirex.

Both Khera _et al_. (1976) and my laboratory found that 1.5 mg mirex/kg/d given during the period of organogenesis in the rat (Days 6.5 or 8.5 to 15.5) had no discernible effects on fetus or newborn (Table 4). However, we recently found that even lower doses, when administered to the mother during the late fetal period (Days 15.5 to 21.5), can induce significant incidences of cardiac problems and perinatal death. The dose of 1 mg/kg/d given during this period of maturation was a total of 8 mg/kg, 2% of the single-dose LD_{50}. It was sufficient to cause a perinatal death rate of 27% (Table 4). Both 1° and 2° heart blocks were present in the ECGs of those who died after birth. Interestingly, several survivors of this experiment continued to exhibit 1° heart blocks for up to 5 d after birth (Grabowski 1982), including some pups who did not develop this problem until after birth.

A group of rats was then given a dose of 0.5 mg/kg/d during this late period. Again, to our surprise, the incidence of perinatal death and dysrhythmias was high. We

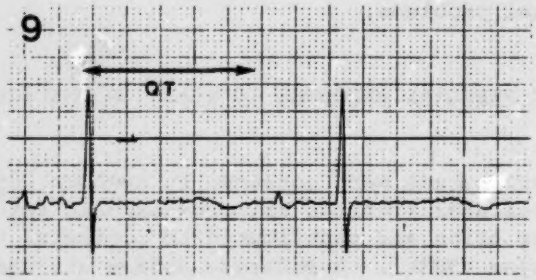

Fig. 9. Prolonged QT interval in the ECG of a rat pup 20 h old, exposed prenatally to 6 mg mirex/kg/d. The rate of heartbeat is 194/min and the QT interval is 0.20 s, twice normal for this age. The pup at this time appeared to have been rejected by its mother and was cannibalized that evening. Chart speed was 125 mm/s. [From Grabowski, Payne 1983b. Used by permission.]

are now administering 0.25 mg/kg/d, a total dose that is only 0.5% of the single-dose LD_{50}, and are still observing a high frequency of problems (Tables 4 and 5). A full 5 d after birth, the mean heart rate (301 beats/min) was significantly lower than that of controls (331 beats/min). Even more interestingly, the range of heart rate in the treated group extended from 72 to 420 beats/min, so the pups at these extremes could be considered as having bradycardia (Fig. 10) or tachycardia. The PR and QT intervals were also longer in the treated groups. As many as 29% of these 5-d-old survivors had PR intervals that would be classified as 1° heart blocks (Fig. 11), and two pups had grossly irregular beats that appeared to be caused by wandering pacemakers.

These observations demonstrate that prenatal exposure to mirex can induce high incidences of dysrhythmias that can persist into the postnatal period. These problems are sufficiently severe to cause some fetal deaths. Some of the milder arrhythmias can persist for several days, while

Table 4. Mortality Rates from Prenatal Mirex Treatment at Two Time Periods

Dosage	Percent of LD_{50}	Perinatal Death Rate (%)
Organogenesis[a]		
Oil-Fed Controls	-	8
3.0 mg/kg/d	7	47
1.5 mg/kg/d	3.5	4
Maturation[b]		
Oil-Fed Controls	-	1.5
1 mg/kg/d	2	28
0.5 mg/kg/d	1	27
0.25 mg/kg/d	0.5	8
0.1 mg/kg/d	0.2	0

[a] Days 8.5 to 15.5.

[b] Days 15.5 to 21.5.

Table 5. Postnatal Effects of Prenatal Exposure to 0.25 mg Mirex/kg/d During Late Gestation: 5-d-Old Pups[a]

	Oil-Fed Controls (N = 40)	Treated (N = 74)
Heart Rate (beats/min)	331 ± 28	301 ± 54
PR Interval (s)	0.059 ± 0.002	0.071 ± 0.035[b]
QT Interval (s)	0.097 ± 0.007	0.103 ± 0.015[c]
Weight (g)	12.2 ± 0.9	12.6 ± 2.4

[a] Values shown are mean ± SD.
[b] Significantly different, $P < 0.01$.
[c] Significantly different, $P < 0.03$.

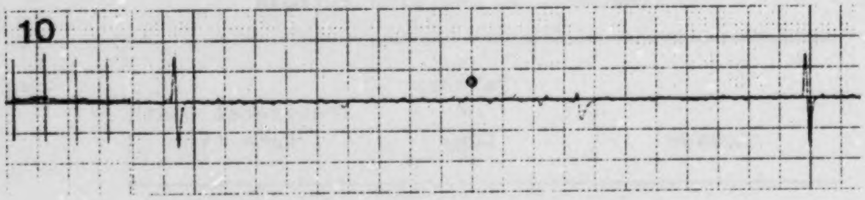

Fig. 10. An electrocardiogram of a 5-d-old rat pup with acute bradycardia. It was prenatally exposed to 0.25 mg mirex/kg/d during the late fetal stages. Segments of the record obtained at 5 and 100 mm/s are shown. The heart rate is 72 beats/min, the PR interval is greatly prolonged, and there is a suggestion of atrial fibrillation. Compare this tracing with that of Fig. 3, which is of a 5-d-old control pup.

others develop after birth. The late fetal period of maturation is more sensitive to this effect of mirex than is

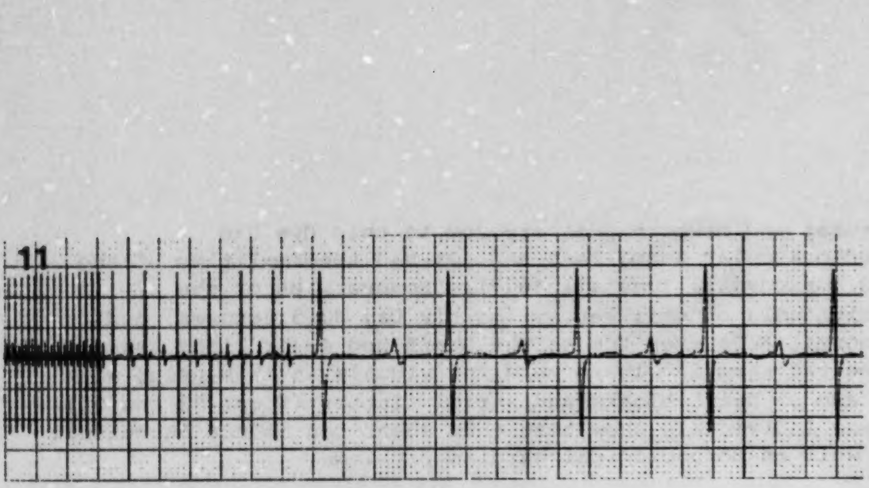

Fig. 11. An ECG of a 5-d-old pup with a persistent 1° heart block. It was prenatally exposed to 0.25 mg mirex/kg/d. The PR interval is 0.10 s, almost twice normal for this age (Fig. 5). Chart speeds: 5, 25, and 100 mm/s.

the period of organogenesis (Grabowski, Daston 1983), which is the traditional time period for application of substances for teratogenic testing.

The kinds of dysrhythmias obtained suggest that the atrium and/or conducting bundles of these immature hearts are being affected by this prenatal toxic exposure. This seems to be a specific effect on immature hearts, because mirex is not known to be either cardioteratogenic or cardiotoxic. The low dose at which these effects can be induced is also surprising. Some effects have been observed in adult rats fed 5 ppm mirex in food (a dose of approximately 0.3 to 0.7 mg/kg/d). These were only minor effects on the liver and reproduction, and they occurred only after at least 28 d of exposure (Chu *et al*. 1981; Yarbrough *et al*. 1981).

DYSRHYTHMIAS INDUCED BY PRENATAL EXPOSURE TO OTHER TOXIC SUBSTANCES

We have tested five other substances for their capacity to induce dysrhythmias: the teratogenic dye trypan blue; aspirin; lithium; ethylenethiourea; and Dipterex, an organophosphate insecticide.

Trypan blue is a well-known, potent cardiovascular teratogen. It is not surprising, then, that 23% of the fetuses and newborn pups exposed to this dye had cardiovascular anomalies, and 12% had abnormalities of the ECG (Grabowski, Tunstall 1977). However, 8% of the individuals of this series (mostly Day 19.5 fetuses) had abnormal ECGs even though the heart and major blood vessels appeared normal. There were individuals in this group with 1° and 2° heart blocks and atrial flutter (Figs. 12 and 13). These individuals show that this agent can induce functional as well as morphological cardiac problems.

Ethylenethiourea, like mirex, is an inducer of edema and not known to be a cardiac teratogen (Khera 1973; Ruddick, Khera 1975). Twenty litters were treated with either a single dose of 250 mg/kg on Day 18.5 or 40 mg/kg/d on Days 7.5 to 18.5. All fetuses were examined on Day 20.5. Several cases of 1° heart block and single cases of 2° block and atrial flutter were found (Figs. 14 and 15). The incidence of problems was low, but significant because comparable problems were never found in any control animals.

The aspirin experiment was a smaller series in which 5 rats were given a single dose of sodium salicylate (625 mg/kg) on Day 10.5 of gestation. Four of the 62 fetuses that were examined on Day 18.5 had 1° blocks, 4 had inverted QRS complexes, and 2 had supernumerary P waves occurring in a random pattern, which suggested a wandering pacemaker.

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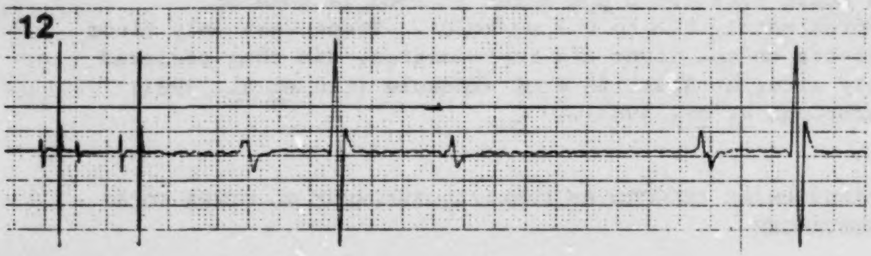

Fig. 12. An ECG of a 19.5-d-old rat fetus with a 2° heart block. The mother was injected with 1 ml of a 0.1% solution of trypan blue on Day 8.5 of gestation. Chart speeds: 25 and 125 mm/s.

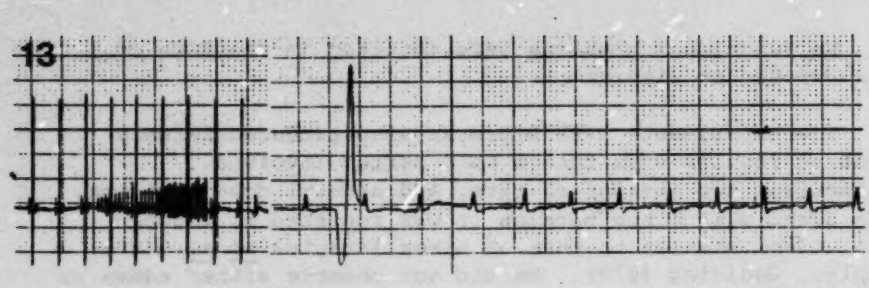

Fig. 13. An ECG of a 19.5-d-old rat fetus that was having occasional periods of atrial flutter. It was exposed to trypan blue as was the fetus in Fig. 12. Chart speeds: 5 and 125 mm/s.

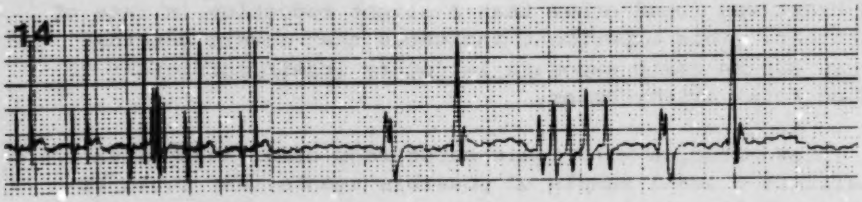

Fig. 14. Periodic episodes of apparent atrial flutter in a 20.5-d-old rat fetus whose mother was injected with 250 mg ethylenethiourea/kg 2 d earlier. Chart speeds: 25 and 125 mm/s.

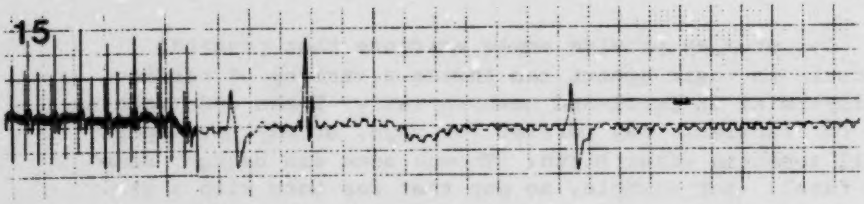

Fig. 15. A 2° heart block (2:1) in a 20.5-d-old rat fetus whose mother was injected with 250 mg ethylenethiourea/kg 2 d earlier. Chart speeds: 5 and 125 mm/s. (The waviness in the baseline is due to 60-cycle electrical interference.)

No functional problems were detected in the ECGs of rats exposed to lithium.

The experiments with Dipterex also produced largely negative results even though this series involved 30 females, two strains of rats, and several dose regimens. This agent was chosen because it was reported to induce fetal edema similar to that of mirex (Staples et al. 1976; Staples, Goulding 1979). We did not observe either edema or dysrhythmias, but this experiment was instructive because it serves as a positive control. The results from one of these series are summarized in Table 6. Dipterex was administered 3 times daily either during the period of organogenesis or the period of maturation. This dose regimen was sufficiently toxic to kill 29% of the dams and to reduce the weight of the fetuses of surviving mothers. No effect on the heart was found other than a slight reduction in rate of heartbeat. This experiment indicates that severe maternal stress due to toxic exposure is not by itself sufficient to induce fetal dysrhythmias.

These studies with toxic substances other than mirex constitute a small sample of possible agents that could be tested. Collectively, they show that the capacity to induce dysrhythmias is not an exclusive property of mirex. It is also apparent that toxic exposure per se, even to a severe extent, does not necessarily induce cardiac problems in the fetus.

CONCLUSIONS

Our studies provide ample evidence that prenatal exposure to toxic agents can induce a variety of cardiac dysrhythmias in fetal and newborn rats. These dysrhythmias may be transient, may persist for days, or may not appear until sometime after birth. Though some are benign, some are fatal. For example, no pup that was born with a 2° heart block survived more than an hour. Since such dysrhythmias are common in human babies, these observations immediately suggest that perhaps some of the dysrhythmias seen in human babies may be caused by prenatal toxic exposure. They also provide an animal model both for study of the etiology of dysrhythmias as well as for fundamental studies on the development of such pathological physiology.

Table 6. Effects of Dipterex (480 mg/kg/d)[a] on Rat Fetuses at Day 20.5 of Gestation

	Number	Heart Rate (beats/min)	PR Interval (s)	Weight (g)
Controls	58	177 ± 31	0.067 ± 0.007	3.3
Dipterex (Days 6.5 to 15.5)	89	161 ± 32[b]	0.072 ± 0.10[b]	3.2
Dipterex (Days 13.5 to 20.5)	50	154 ± 27[b]	0.068 ± 0.006	3.0[b]

[a] Maternal mortality was 29%.

[b] Significantly different, $P < 0.01$.

Mirex is, to date, the most prolific inducer of these problems, but it is not the only substance that can do this. Of those producing positive results, trypan blue and aspirin are known cardiac teratogens and hence might have been suspect, but neither mirex nor ethylenethiourea have been previously suspected of having any kind of effect on fetal hearts. The discovery of their cardiac effects was fortuitous.

The effects of mirex, at least, seem to be specific for the fetal heart, because maternal hearts do not respond in the same fashion, even at moderate dose levels. The production of significant incidences of dysrhythmias by very low doses of mirex and the demonstration that near-lethal doses of an organophosphate insecticide (Dipterex) do not induce dysrhythmias support this hypothesis.

Finally, these studies demonstrate the usefulness of functional testing to evaluate developmental toxicity. It was previously known that mirex exposure can induce a high perinatal death rate, but the cause of these deaths was not known. The cardiac problems are not accompanied by any visible abnormalities. The use of the ECG was the only way in which these dysrhythmias could have been detected. The use of the electrocardiogram was also the only way in which the problems induced by very low doses of mirex could have been detected, because these are not necessarily accompanied by high death rates. Using conventional criteria, a dose of 1.5 mg mirex/kg for 6 to 8 d during organogenesis was accepted as a "no obvious effect level" (Khera et al. 1976; Grabowski unpublished). Using physiological criteria and dosing during the period of maturation, a no-effect level has yet to be determined, but appears to be at least one order of magnitude lower. This demonstrates the greater sensitivity of the functional test, in this instance, to detect developmental toxicity.

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ELECTROCARDIOGRAPHIC STUDIES OF DEVELOPING RODENTS EXPOSED IN UTERO TO TRYPAN BLUE

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INTRODUCTION

Abnormal development of the heart and vascular system may result from a multiplicity of causes. Often, such abnormalities may be correlated with changes in fetal environmental conditions or *in utero* exposure to toxic or teratogenic substances (Grabowski 1977; Jaffee 1977; Jaffee 1978; Wilson 1960). It is therefore important to test the impact of suspected harmful agents or conditions on the viability of the developing animal in order to more accurately predict the potential consequences following similar exposure in humans.

Traditional research methods of screening compounds or conditions for fetotoxic and/or teratogenic potential rely primarily on gross anatomical examination of the fetus or neonate. While these methods are important and provide much useful information, they possess several inherent limitations with regard to the cardiovascular system, namely a lack of sensitivity and an inability to obtain multiple data from an individual animal.

With regard to the first point, a positive response to the experimental insult is dependent upon the detection of anatomical malformations. To produce such grossly visible defects, it is often necessary to utilize uncommonly high levels of exposure to the experimental treatment. Such defects may in fact represent extreme examples of abnormal development. Subtle anatomical variations resulting from exposure to lower, more realistic levels of teratogenic agents might be overlooked using these methods (Balazs, Ferrans 1978). Such subtle variations, while not life threatening, may also compromise cardiovascular function, particularly during situations of physiological stress (Balazs 1972).

With respect to the second point, the detection of anatomical defects in the cardiovascular system requires sacrifice of the animal. In many experimental animal models, substantial development takes place during the neonatal period. The ability to make repeated observations and acquire multiple data from treated animals is thus potentially advantageous in order to assess the effect of exposure on continued postnatal development. This is impractical to do when relying solely on anatomical criteria.

The use of functional parameters to monitor abnormal developmental processes may offset the above limitations (Zbinden 1981). The cardiovascular system is uniquely suited to the measurement of such parameters, and the development of methods to assess the functional cardiovascular capability of fetal and neonatal animals should substantially augment traditional methodology. Thus, it may be possible to detect subtle developmental abnormalities by evaluating cardiac function following treatments that employ lower, more appropriate levels of exposure. In addition, neonates may be routinely examined with non-invasive methods throughout the course of development, thereby minimizing experimental animal requirements and facilitating trend analysis. Due to the increased sensitivity of the testing procedures and the importance of the cardiovascular system to systemic well-being, such methodology should permit better characterization of the extent of the toxic or teratogenic insult to the animal. For example, there are developmental problems that do not manifest themselves as overt malformations, such as conduction defects and biochemical abnormalities, which may exert a significant impact on an animal's viability or response to stress. Conversely, there may be significant anatomical variations that do not adversely affect functional performance.

The focus of our experimental effort has been to develop methodology and applications designed to evaluate abnormal cardiovascular function in fetal and neonatal rodents. The experimental approach adopted to accomplish these goals involves a three-tiered protocol based on analyses of standard cardiovascular parameters. Specifically, the three tiers or phases consist of 1) a screening procedure, 2) an isolated heart procedure, and 3) an in vivo analysis of multiple cardiovascular parameters. The screening procedure is based on a precise, one-dimensional, computer-assisted analysis of the electrocardiogram (ECG). It is a relatively atraumatic procedure, permits a variety of experimental animal models and preparations, and allows repeated measures of individual animals (Watkinson et al. 1982). The isolated heart procedure utilizes a Langendorff or Morgan apparatus, which allows the measurement of additional functional indices such as pressure development and cardiac output. It also permits a more rigorous evaluation of direct effects of substances on cardiac function in the absence of mitigating systemic influences, such as cardiac reflexes and circulating hormones. The in vivo procedure provides a more physiological extension of the isolated heart preparation. By measuring such parameters as systemic blood pressure, dP/dt, cardiac output, and regional blood flow in the intact animal, it should be possible to more accurately describe cardiovascular function.

This paper will report on the application of developed electrocardiographic analytical procedures to fetal and neonatal animals exposed in utero to the teratogenic dye, trypan blue. Trypan blue was selected as an appropriate agent to test the applicability of these procedures to the study of abnormal cardiovascular development for several reasons. First, in utero exposure to trypan blue has been shown to produce significant cardiovascular anomalies in the developing rat fetus (Fox, Goss 1958; Grabowski, Tunstall 1977; Monie et al. 1966). Although trypan blue exposure also produces defects in other organ systems, these defects are either less serious (e.g., ocular defects) or infrequently observed (e.g., central nervous system defects). Thus, the cardiovascular system appears to be the organ system most sensitive to teratogenesis induced by trypan blue. Second, it has been reported that a high percentage of animals exposed to trypan blue survive postnatally for extended periods of time with serious cardiovascular malformations (Richman _et al_. 1957). Hence, a large population of animals would be available for postnatal analysis. Thus, it was felt that the serious nature of the defects produced, in conjunction with the relative nonlethality obtained, would facilitate detection of cardiac abnormalities with these analytical procedures in both fetal and neonatal animals.

METHODS

Animals

Pregnant 90-day-old CD rats (Charles River Breeding Laboratory, Kingston, NY) were used in this study. All animals were housed individually in plastic cages on pine-shaving bedding in a temperature (22-22°C) and light controlled (12 h light:12 h dark) bio-clean room. Throughout the study, animals received tap water and laboratory chow _ad libitum_.

Protocol

Animals were randomly assigned to three treatment groups. Animals in the low-dose and high-dose treatment groups were injected intraperitoneally on Day 9 of gestation with 0.5 ml and 1.0 ml, respectively, of a 1% solution of trypan-blue dye (Eastman Kodak Co.; 92% pure) dissolved in normal saline. Control animals received 1.0 ml of normal saline. Fetuses from 30 animals (10 per treatment group) were studied on Day 21 of gestation. The offspring of the remaining 48 animals (16 per treatment group) were studied on postnatal Days 1 through 10.

Experimental Procedure - Fetal

On Day 21 of gestation, dams were lightly anesthetized with methoxyflurane and injected with sodium pentobarbitol

(36 mg/kg; IP). A midline abdominal incision was made and the gravid uterus was exposed and examined to determine the number of implantations and the number of viable fetuses. Fetuses were carefully exteriorized so that all placental and vascular connections remained intact. Each fetus was placed on a paraffin retaining tray and four ECG electrodes (IN VIVO METRICS, Heraldsburg, CA) were positioned near the juncture of the limbs (Robinson et al. 1982). Leads I, II, and III of the ECG were monitored for approximately one to two minutes and recorded on magnetic tape. The fetus was then removed from the mother and examined for visible anatomical defects.

A total of 146 fetuses were monitored (42, 43, and 61 from the control, low-dose, and high-dose groups, respectively). These animals were later dissected and examined for gross morphological abnormalities of the heart and great vessels.

Experimental Procedure - Postnatal

With the exception of slight modifications, the experimental procedure followed for the postnatal animals was identical to that observed for the prenatal animals. Pups were unanesthetized and lightly restrained. Four needle electrodes (Grass Instrument Company, Quincy, MA) were positioned at the juncture of the limbs. Leads I, II, and III of the ECG were monitored and recorded on magnetic tape from approximately 40 pups per day on postnatal Days 1 through 10. At least one pup of each sex was randomly selected from each litter (n = 6 to 8) for each treatment group. Animals were examined for grossly visible external anatomical defects but were not dissected and examined for cardiovascular malformations.

Electrocardiographic Analysis

Electrocardiographic analysis was conducted using a computer-assisted analysis system (Watkinson et al. 1982). Briefly, this procedure allows the operator to screen the waveform for arrhythmias and employs a waveform ensembling technique to provide a one-dimensional analysis of ECG intervals.

Statistical Analysis

Statistical analysis was conducted using the general linear models procedure of the Statistical Analysis System (SAS Institute, Inc. 1982). The least-square means procedure was used to test for differences between groups. Probability values less than 0.05 were considered significant.

RESULTS AND DISCUSSION - FETAL

Teratology Analysis

In utero exposure to trypan blue produced a substantial number of teratogenic effects in Day 21 fetuses, such as decreased viability, external anatomical defects, and malformations of the heart and great vessels. These effects are summarized in Tables 1 and 2.

There was no difference in the number of implantations among groups; however, the number of live fetuses on Day 21 was significantly decreased in both trypan blue treated groups (Table 1). Although there was no significant difference in fetal weight among the groups, this could possibly have been an experimental artifact due to the

Table 1. Implantations and Litter Sizes Found in Fetal Study

Group	No. Implantations (No. Litters)	No. Live Fetuses (No. Litters)
Control	11.6 ± 1.0[a] (7)	11.4 ± 1.1 (7)
Low Dose	11.5 ± 0.6 (6)	8.8 ± 1.5[b] (6)
High Dose	11.9 ± 0.9 (8)	8.0 ± 0.8[b] (8)

[a] Values are mean ± S.E.M.

[b] Indicates statistical significance ($P<0.05$).

Table 2. Defects Found in Fetal Study[a]

Type	Number
Noncardiac Defects	
Exencephaly	10
Encephalocele	3
Umbilical Hernia	3
Fused Hindlimbs	1
Tail Hematoma	1
Cardiac Defects	
Brachiocephalic Artery Defect	9
Malpositioned Great Vessels	4
Right Atrial Transposition	4
Left Atrial Transposition	3
Aortic Arch Defects	2
Dextrorotated Heart	2
Right Subclavian Artery Defect	2
Small Atria	1
Enlarged Heart	1
Pulmonary Artery Defect	1
Right Superior Vena Cava Defect	1
Septal Defect (suspected)	6

[a] A total of 150 fetuses were examined, of which 42 were from the control group, 45 were from the low dose trypan blue treated group, and 63 were from the high dose trypan blue treated group.

smaller litter sizes. Such a positive growth effect due to fewer numbers of fetuses per litter would potentially augment viability both prenatally and postnatally.

Noncardiac defects (Table 2) include all grossly visible external terata. Fetuses were not dissected or rigorously examined for morphological abnormalities to organ systems other than the cardiovascular system. With the exception of a slight tail hematoma, no terata were observed in fetuses from the control group. Approximately 8.9% of the fetuses examined in the low dose treatment group were visibly deformed, while 20.6% of the fetuses in the high dose treatment group exhibited terata.

Cardiac defects (Table 2) include all grossly visible malformations of the heart and vasculature observed following postmortem mid-sternal thoracotomy. More subtle defects, such as small septal fistulas and valvular anomalies, were not rigorously investigated. One fetus from the vehicle-treated control group (n = 42) was found to have a dextrorotated heart. The remaining malformations were found in fetuses from the trypan blue treated groups (n = 108). A total of 36 defects was found in 27 fetuses. Thus, 22.2% and 27.0% of the treated animals in the low-dose and high-dose groups, respectively, developed cardiovascular malformations. While the percentages of fetuses with cardiovascular malformations were similar in both groups, it is noteworthy that three of six litters examined from the low-dose group were found to have at least one fetus with a malformation of the heart or great vessels, while seven of eight high-dose litters had at least one fetus with a cardiovascular malformation.

Electrocardiographic Analysis

A total of 146 fetuses (42, 43, and 61 from control, low-dose, and high-dose groups, respectively) were monitored electrocardiographically. Waveforms were first evaluated for arrhythmias and qualitative differences from normal rat ECG patterns. Interval and heart rate (HR) analysis was conducted using an automated procedure (Watkinson et al. 1982).

No arrhythmias were observed in any of the recorded fetal ECGs. Although a large number of irregular waveforms were found in all groups, there was substantially more variability in those of fetuses from the trypan blue treated groups (Table 3). Twenty-four qualitatively atypical ECG patterns were found in 13 different fetuses in the control group, 50 irregularities were found in 25 fetuses in the low-dose group, and 50 irregularities were found in 20 fetuses in the high-dose group (Table 4). Thus, in utero exposure to trypan blue resulted in an increase in the total number of ECG irregularities observed as well as an increase in the number of animals with atypical ECGs. Using these data it is possible to calculate the average variability per

Table 3. Electrocardiographic Irregularities Found in Fetal Study[a]

Irregularity	Number
Large T-Wave	41
Elevated ST-Segment	34
Depressed PR-Segment	22
Negative R-Wave/Inverted QRS Complex	12
Biphasic P-Wave	6
Large P-Wave	5
Negative P-Wave	3
Elevated PR-Segment	1

[a] A total of 146 fetuses were monitored, of which 42 were from the control group, 43 were from the low dose trypan blue treated group, and 61 were from the high dose trypan blue treated group.

group (no. irregularities per group/no. animals with irregularities in that group). The values obtained for the control, low-dose, and high-dose groups were 1.85, 2.00, and 2.50, respectively. Thus, there is a dose-related increase in the degree of ECG variability per atypical animal following *in utero* exposure to trypan blue. By correlating the morphological defects with the observed ECG irregularities in each group, it can also be demonstrated that there is a dose-related increase in the incidence of fetuses with both anatomical and electrocardiographic abnormalities.

While it is possible to demonstrate qualitative treatment-related differences in ECG waveforms, quantitative analysis yielded no consistent differences in HR or

Table 4. Morphological Defects and Electrocardiographic Irregularities Found in Fetal Study

Group	No. Fetuses (No. Litters)	No. Morphological Defects (No. Fetuses with Defects)	% Fetuses with Defects	No. ECG Irregularities (No. Fetuses with Irregularities)	% Fetuses with Irregularities
Control	42 (7)	2 (2)	4.7%	24 (13)	30.9%
Low Dose	43 (6)	17 (14)	32.6%	50 (25)	58.1%
High Dose	61 (8)	35 (30)	49.1%	50 (20)	32.8%

intervals among the groups. This may be attributable, in part, to several faults in the original design of the study. Although trypan blue is a proven cardiovascular teratogen (Fox, Goss 1958; Grabowski, Tunstall 1977; Monie et al. 1966), it does not produce consistent anatomical malformations (Table 2). Therefore, while many of the malformations may be associated with ECG irregularities per se, they may affect different aspects of the waveform and interval analysis. This diverse effect on ECG intervals would be further compounded by the statistical analysis procedure, which averages the interval data within each group. Thus, it is possible to obscure true effects that may have been present in individual waveforms. Additionally, although many anatomical defects were observed in fetuses exposed in utero to trypan blue, it is highly probable that the most functionally impaired fetuses did not survive. Most of the fetuses in the treated groups were either anatomically normal or exhibited relatively minor defects. The smaller litter sizes probably further contributed to increased viability of the treated animals. Thus, the relatively large number of functionally normal animals, coupled with the averaging of interval values, would further contribute to the masking of true quantitative effects. Finally, the effects of anesthesia on ECG interval analysis in the fetal rat are unclear and were not completely accounted for by the study design. It is possible that there could have been an interaction between the anesthesia and the treatment effects in either the dams or fetuses in the trypan blue exposed groups.

RESULTS AND DISCUSSION - POSTNATAL

Teratology Analysis

Data obtained from analysis of postnatal animals were qualitatively similar to those of fetal animals. Litter size in the trypan blue treated groups on postnatal Day 1 was reduced from that seen on gestational Day 21 in the fetal animals and was significantly smaller than the litter size found in postnatal control animals (Table 5). There was no difference in neonatal weight among the groups when measured on postnatal Day 1. This was most likely attributable to the reasons discussed above.

Table 5. Litter Sizes Found in Postnatal Study

Group	Live Fetuses (No. Litters)[a]
Control	11.2 ± 0.6 (8)
Low Dose	6.2 ± 0.9[b] (6)
High Dose	5.0 ± 0.8[b] (11)

[a] Values are mean ± S.E.M.
[b] Indicates statistical significance ($P<0.05$).

Noncardiac defects (Table 6) were essentially similar to those found in fetuses. No terata were observed in animals from the vehicle-treated control group, while 22.0% of the treated animals examined exhibited visible defects, the major percentage of which were found in the high-dose group (5.4% and 33.3% for the low- and high-dose groups, respectively). It should be noted that all fetuses were examined for noncardiac defects on postnatal Day 1. Other defects which were not apparent at that time but were observed later are not included in Table 6. All defects observed after postnatal Day 1 were found in treated animals.

Viable postnatal animals were not examined for cardiac defects; however, the percentage of cardiovascular malformations was most likely lower in the postnatal treated groups as severely defective animals probably did not survive. A small group of animals (n = 11), which were either stillborn or died within the first few days, was dissected and examined. Some of the more severe cardiovascular malformations were found in these animals.

Electrocardiographic Analysis

Analysis of ECG in postnatal animals was conducted as described above for fetal animals. The number of ECGs

Table 6. Noncardiac Defects Found in Postnatal Study[a]

Type	Number
Encephalocele/Microencephalocele	6
Exencephaly	4
Hindlimb Paralysis	1
Rudimentary Tail	1
Imperforate Anus/Missing External Genitalia	1
Eye Defect	1
Facial/Cranial Hematoma	6

[a] A total of 172 animals were examined, of which 81 were from the control group, 37 were from the low dose trypan blue treated group, and 54 were from the high dose trypan blue treated group.

monitored in animals from the control, low-dose, and high-dose groups on postnatal Days 1 through 10 were 126, 128, and 144, respectively.

Irregularities observed in postnatal ECG analyses are summarized in Table 7. No arrhythmias were found in animals in the control group; however, a few abnormal rhythms/beats were noted in treated animals. As in the fetal study, there was a dose-related increase in the number of ECG irregularities observed as well as in the number of ECG irregularities associated with terata (Table 8).

Analysis of HR and intervals demonstrated no consistent differences among groups, most likely for the same reasons discussed above. The decreased survival rate of functionally impaired animals is perhaps more applicable in the postnatal animals, as shown by the decrease in litter size when compared with the litter size observed on

Table 7. Electrocardiographic Irregularities Found in Postnatal Study[a]

Type	Number
Negative R-Wave/Deep S-Wave/ Inverted QRS Complex	106
Small/Negative P-Wave	95
Small/Negative T-Wave	82
Elevated ST-Segment	47
Depressed ST-Segment	40
Biphasic/Double P-Wave	31
Large T-Wave	30
Depressed PR-Segment	21
Wide QRS Complex	18
Large P-Wave	11
Arrhythmia	9

[a] Data were obtained from a total of 399 electrocardiograms, of which 126 were from the control group, 128 were from the low dose trypan blue treated group, and 144 were from the high dose trypan blue treated group.

gestation Day 21. Thus, a smaller number of abnormal animals would be included in the averaging procedure.

Table 8. Morphological Defects and Electrocardiographic Irregularities Found in Postnatal Study

Group	No. Observations (No. Litters)	No. ECG Irregularities	No. ECG Irregularities Associated with Terata
Control	126 (16)	86	0
Low Dose	128 (16)	177	37
High Dose	144 (16)	227	55

SUMMARY

Techniques, based on computer-assisted analyses of ensembled ECG waveforms, have been developed in this laboratory to serve as a screening procedure for potentially cardiotoxic substances. This procedure was tested on fetal and neonatal rats exposed *in utero* to two levels of trypan blue, a known cardiovascular teratogen. The offspring of the treated animals exhibited decreased viability, as well as significant levels of both cardiac and noncardiac malformations. Qualitative assessment of ECG irregularities demonstrated an increased number of abnormalities in the treated groups. Correlations were also obtained between observed morphological defects and ECG irregularities in the trypan blue treated groups.

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CARDIOTOXICITY OF PERINATAL LEAD EXPOSURE

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INTRODUCTION

Many reports have appeared in the literature detailing the acute and chronic toxicities of Pb directed at various organ systems, including the cardiovascular system (Freeman 1965; Kline 1960; Myerson, Eisenhauer 1962; Read, Williams 1952). Various studies have focused on the effects of Pb exposure on animals during the neonatal period. It is clear that absorption of Pb in neonates is more rapid and more complete than in adult animals (Kello, Kostial 1973). However, it has also become apparent that some long-term effects are produced during this period that cannot be completely explained by either the concentration of Pb achieved in the organ in question, or the persistence of Pb in tissues. Most of the observations of persistent Pb effects have concerned some function of the central nervous system (CNS). For example, the exposure of neonatal rats to Pb has been shown to cause learning deficits measured at some time after termination of exposure (Brown 1975). In these experiments an exposure window was identified, suggesting that an effect on development was involved. Long-lasting effects on the visually evoked cortical response in rats have also been demonstrated after early Pb exposure (Fox et al. 1977). The remainder of this communication will deal with an effect of early neonatal Pb exposure on the function of the heart, measured 3 months after termination of exposure.

EXPOSURE OF ANIMALS

Albino rats of both sexes were exposed to Pb indirectly by providing a 0.2% Pb acetate solution to lactating dams as their only drinking solution. Pups were thus exposed to Pb for all or part of the nursing period (21 d). Exposure to Pb at this concentration had no effect on growth or on the time of appearance of developmental landmarks, i.e., eye opening and fur appearance, as compared with control pups similarly administered sodium acetate. The one observable difference between Pb-exposed and control animals was the general irritability and aggressiveness of treated animals.

TESTING OF CARDIAC EFFECTS

Two models of cardiac testing were used. Cardiac function was tested *in vivo* using rats anesthetized with a combination of pentobarbital (20 mg/kg) and urethane (240 mg/kg). Animals were artificially respired, and polyethylene cannulae were inserted into a carotid artery and a jugular vein for recording systemic blood pressure and for drug injection, respectively. Pin electrodes were positioned for recording the limb lead II electrocardiogram. In the second model, hearts were removed and attached to the outlet of a perfusion column for retrograde perfusion by the Langendorf procedure.

LEAD-ENHANCED ARRHYTHMOGENIC RESPONSE TO NOREPINEPHRINE

Cardiac arrhythmias are a well-documented feature of the toxic response to high concentrations of norepinephrine (NE). Random premature ventricular contractions (PVCs) are not uncommonly seen as random events during infusions of NE at a rate of 80 $\mu g/kg\ min^{-1}$ to rats. When this same NE infusion was administered to adult rats (3 to 4 months old) that had been exposed to Pb throughout the 21-d nursing period, PVCs occurred at a rate approximately 4 times that seen in control animals (Fig. 1) (Williams *et al*. 1977). Despite the greater apparent sensitivity of the heart to NE, the blood-pressure response of Pb-exposed animals was no different from that seen in control animals. Administration of graded doses of NE by bolus injections caused similar pressor responses in control and Pb-exposed rats, but it was more likely to cause cardiac arrhythmias in Pb-exposed rats

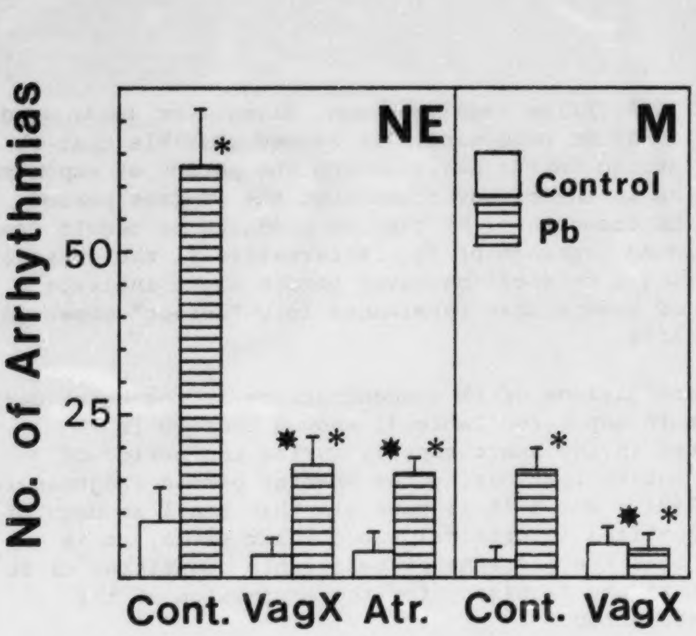

Fig. 1. Arrhythmogenic effects of NE and methoxamine (M) in Pb-exposed and control (Cont.) rats. Anesthetized rats were given equipressor infusions of NE (80 μg/kg min^{-1}) or M (800 μg/kg min^{-1}) before or after bilateral vagotomy (VagX) or atropine (Atr.) (1 mg/kg) administration. Data are expressed as number of ventricular extrasystoles occurring during a 2-min infusion. Asterisks denote values for Pb-exposed animals that differ significantly from corresponding controls. Stars indicate values after atropine or vagotomy that significantly differ from pretreatment values.

than in control rats. Even with the lowest doses used, which caused no arrhythmias in control rats, Pb-exposed rats developed cardiac arrhythmias.

LEAD ACCUMULATION

Two explanations could be suggested for the observed Pb-induced cardioxtoxicity. Cardiac conduction abnormalities have been infrequently observed as a part of the syndrome of experimental (Kopp et al. 1978) and clinical

(Freeman 1965; Kline 1960; Myerson, Eisenhauer 1963; Read, Williams 1952) Pb poisoning. It seemed possible that Pb might be stored in the heart during the period of exposure and persist in the tissue throughout the Pb-free period. The effects observed might then be proposed to result from the continued presence of Pb. Alternatively, the presence of Pb during a critical neonatal period might initiate a sequence of events that terminates in a "defect" observed later in life.

Determinations of Pb concentrations in the heart during and after Pb exposure (Table 1) showed that Pb is accumulated in the heart rapidly during the period of exposure but is lost during the Pb-free period (Hejtmancik *et al*. 1982). While it is possible that small amounts of Pb remain localized in critical subcellular sites, it is apparent that the presence of measurable quantities of Pb in the heart is not necessary for the expression of the cardiotoxic effect.

Table 1. Tissue Pb Concentrations and NE Arrhythmogenesis

		Pb Concentration		
Experiment	Exposure Time (d)	Blood (μg/dl)	Heart (μg/g)	PVCs During NE Infusion[a]
Pb Pups	1-21	47.20 ± 2.76[b]	3.97 ± 0.61[b]	7.0 ± 1.6[b]
Control	---	4.90 ± 1.10[b]	0.52 ± 0.08[b]	1.8 ± 0.8[b]
Pb Pups	1-10	37.82 ± 1.21[b]	1.71 ± 0.08[b]	59.5 ± 14.4[c]
Pb Pups	11-21	42.40 ± 1.84[b]	2.88 ± 0.64[b]	9.6 ± 4.7[c]
Pb Adults	1-21	5.22 ± 0.64[c]	0.53 ± 0.10[c]	58.0 ± 12.8[c]
Control	---	5.56 ± 0.93[c]	0.42 ± 0.08[c]	11.2 ± 3.2[c]
Pb Dams	1-21	64.96 ± 6.68[b]	2.31 ± 0.02[b]	22.8 ± 7.8[b]
Control	---	3.64 ± 1.91[b]	0.39 ± 0.11[b]	8.9 ± 5.7[b]

[a] 80 μg/kg min^{-1} for 2 min.

[b] Measured at the end of Pb-exposure period.

[c] Measured 99-139 d after termination of exposure.

TIME OF EXPOSURE

To test the possibility that the time of Pb exposure was critical in the production of the cardiac abnormality, additional litters of rat pups were exposed indirectly to Pb during either 1 to 10 or 11 to 21 d after birth. Although the maximum concentration of Pb achieved in the hearts of pups treated on Days 1 to 10 was significantly less than that accumulated in pups treated on Days 1 to 21, the incidences of NE-induced arrhythmias in these animals tested as adults, were approximately the same (Table 1). In contrast, pups exposed to Pb during Days 11 to 21 showed no greater incidence of NE-induced arrhythmias than did control rats (Hejmancik, Williams 1979a).

During Pb exposure, blood-Pb concentrations in pups never exceeded 50 μg/dl, a level that in humans is considered to be in the low toxic range. Blood-Pb concentrations in nursing dams during the exposure period reached levels as high as 65 μg/dl (Table 1). NE administration to dams within 2 weeks of termination of Pb exposure produced a significantly greater number of PVCs than in untreated female rats of the same age, but this number was smaller than that in adult rats exposed to Pb as neonates (Table 1). In dams 3 months after termination of Pb exposure, NE still caused a greater number of cardiac arrhythmias than in untreated rats, but the difference between the two groups at this time was less pronounced than immediately after Pb exposure.

To ascertain if the observed effect of Pb on the heart occurs at the time of exposure or is the result of a Pb-induced change in a process of cardiac development, Pb-exposed and control animals of ages 18 to 120 d were subjected to NE challenge. No differences could be detected in the responses of Pb-exposed and control animals younger than 70 d. However, at 70 d of age and older, Pb-exposed animals developed a significantly greater number of PVCs than did controls.

DIRECT AND INDIRECT ACTIONS OF NE

NE-induced cardiac arrhythmias may occur not only as a result of the direct actions of NE on the cardiac conductive system, but also through autonomic reflexes initiated by the increase in systemic blood pressure. To examine the possibility that indirect effects of NE were involved in the enhanced arrhythmogenic response, two types of experiments were performed. In Pb-exposed rats either atropine pretreatment or bilateral vagotomy attenuated, but did not eliminate, the arrhythmogenic effect of NE (Hejtmancik, Williams 1979b). Methoxamine (M), the alpha adrenergic agonist, is considered to have no direct action on the heart; its effects on the heart should mimic only the indirect component of the action of NE. Both M and NE, when given in equipressor doses, caused significantly more cardiac rhythm disturbances (primarily extrasystoles) in Pb-exposed than in control animals, but M was only approximately half as effective in this action as was NE. Either atropine pretreatment or bilateral vagotomy could eliminate the arrhythmogenic, but not the pressor, effect of M (Fig. 2). The latent cardiotoxicity caused by neonatal Pb exposure thus appears to consist of both a vagal-mediated component and another more direct component. Additional evidence that a direct effect on the heart participates in the exaggerated effect of NE comes from experiments with isolated perfused hearts. Administration of NE caused no greater increase in contractility in Pb-exposed than in control hearts, but disturbances in rhythm were significantly more frequent in hearts from Pb-exposed animals (Fig. 2).

It could therefore be concluded that the presence of Pb in sufficient concentrations during a critical neonatal period in some way altered the course of development such that the change was not apparent until the animals matured, and even some stimulus was required before the change appeared. Further, it seemed that the enhanced response to NE involved at least two separate effects, one of which was directly on the heart; the other was on the autonomic system and was mediated, at least in part, via the vagus nerve.

EFFECTS ON THE PARASYMPATHETIC REFLEX ARC

An effect precipitated by NE that appears to occur through vagal nerve activity could be localized at any site in the vagal reflex arc, affector, central, or effector component. In the experiments with anesthetized intact rats, no difference was seen in the pressor responses of experimental and control rats, indicating the signal to the

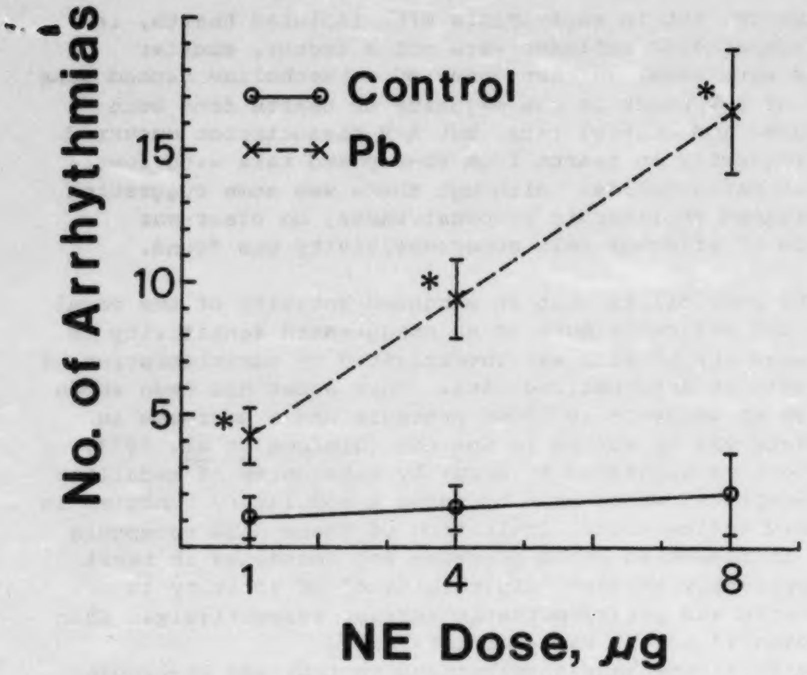

Fig. 2. Arrhythmogenic effect of NE in isolated rat hearts from Pb-exposed and control animals. Data are expressed as number of ectopic beats in 2 min following NE dose. Asterisks denote those values in Pb-exposed hearts that differ significantly from the corresponding control.

affector component was not altered; however, it is possible that the baroreceptors perceive the signal differently. Central connections of the vagal reflex arc may be affected, an action not inconsistent with known CNS sensitivity to Pb. Possible sites of action in the effector component include both the activity of the vagus nerve and the sensitivity of the effector cell. While no experiments have been directed at the determination of vagal nerve activity, some experiments have examined the sensitivity of the effector cell to the neurotransmitter. The effects of graded doses of methacholine were no different in Pb-exposed and control anesthetized rats. The presence of intact sympathetic reflexes complicated the interpretation of these experiments, but in experiments with isolated hearts, in which sympathetic reflexes were not a factor, similar results were seen. Higher doses of methacholine caused some degree of A-V block in the majority of hearts from both Pb-exposed and control rats, but A-V dissociation occurred more frequently in hearts from Pb-exposed rats with lower doses of methacholine. Although there was some suggestion of increased cholinergic responsiveness, no clear-cut evidence of effector cell supersensitivity was found.

The possibility that an enhanced activity of the vagal reflex arc may contribute to an exaggerated sensitivity to NE-induced arrhythmias was investigated by administration of picrotoxin to anesthetized rats. This agent has been shown to cause an increase in blood pressure and a decrease in heart rate via an action in the CNS (Dimicco *et al*. 1977). The effect is suggested to occur by antagonism of medullary GABA receptors, which seem to serve a modulatory function in autonomic reflex arcs. Inhibition of these GABA receptors causes increases in blood pressure and decreases in heart rate apparently through "disinhibition" of activity in sympathetic and parasympathetic nerves, respectively. When picrotoxin (2 mg/kg) was administered to pentobarbital/urethane anesthetized control and Pb-exposed rats, the blood pressure increase was comparable in both groups, but control animals responded with a greater decrease in heart rate than did Pb-exposed animals. If the bradycardia produced by picrotoxin is a result of medullary GABA receptor antagonism, it would appear that little or no GABA inhibition is exerted on the vagal reflex arc in Pb-exposed animals, so that blocking GABA responses with picrotoxin has less effect than in untreated animals. If gabergic transmission is decreased by Pb exposure, the result could be an increase in parasympathetic activity, as seen both in patients (Read, Williams 1952; Myerson, Eisenhauer 1963; Crepet *et al*. 1956) and experimental animals (Stykhinskaya 1965), and could explain the apparent vagal hyperreactivity seen in rats exposed as neonates.

CARDIAC ADRENERGIC FUNCTION

The first 3 postnatal weeks are particularly important in the development of the sympathetic nervous system in rats (Iverson *et al*. 1967). Cardiac NE levels are quite low at birth but rise during this period to adult levels. The development of neurotransmitter uptake sites slightly precedes growth of NE stores. Measures of both uptake capacity and NE content have been used as indices of adrenergic nerve development. The critical period for the production of latent cardiotoxicity after Pb exposure parallels closely the period of rapid development of sympathetic innervation of the heart. It is conceivable that interference with the development of sympathetic nerves might result in altered sensitivity to NE in adulthood. A decrease in ventricular NE concentration has also been observed in young Pb-injected rats (Levina 1973; Chekunova 1974; Chekunova 1978). These observations suggest that Pb might alter the development of adrenergic nerves.

Studies in this laboratory have investigated the effect of neonatal Pb exposure on the development of adrenergic innervation of the heart. The development of biochemical parameters indicative of both presynaptic and postsynaptic sympathetic innervation of the heart was studied. Analyses were performed at 4- or 5-d intervals up to postnatal Day 25 and at longer intervals thereafter.

Presynaptic noradrenergic parameters studied included 1) neuronal uptake of ^{3}H-NE, 2) vesicular storage of ^{3}H-NE, and 3) steady-state levels of NE. Each of these variables shows its own characteristic pattern of development. No significant differences were observed between Pb-exposed and control rats with respect to the gradual development of neuronal uptake of ^{3}H-NE. However, the vesicular storage of ^{3}H-NE was significantly increased (63%) on postnatal Day 4 in the Pb-treated rats, suggesting that Pb exposure may enhance the maturation and/or the axonal transport of NE storage vesicles. The steady-state levels of cardiac NE were also found to be significantly increased (86%) at this early stage of development. It could be anticipated that this transient increase in presynaptic noradrenergic development might result in down-regulation of the β-adrenergic receptor. However, no significant effect of Pb exposure on either the density of receptor sites or the affinity of the receptor for the radioligand, ^{3}H-dihydroalprenolol, could be detected (Abreu, Williams 1982). Despite the lack of an observable effect on β-receptor activity, NE-stimulated adenylate cyclase activity was significantly decreased (approximately 10%) in Pb-treated rats from postnatal Day 15 through Day 25. Basal and NaF-stimulated cyclase activity showed similar decrements at these points. Interestingly, the most pronounced effect of Pb exposure on β-receptor coupled cyclase activity (30% decrease in maximal response to NE) was detected in animals that were 16 to 19 months old. The ED_{50} for NE stimulation of cardiac cyclase activity was concomitantly increased by approximately 70% in these "old" Pb-exposed rats as compared with age-matched controls. While these data do not explain the observed sensitivity of the Pb-exposed heart to NE, they do indicate a persistent effect of early Pb exposure that becomes more apparent as animals age.

SPECIFICITY OF THE CARDIOTOXIC EFFECT

Although ouabain and procainamide are considered to act in different ways on the heart, both produce cardiac arrhythmias at toxic doses. Both drugs were given by intravenous infusion to anesthetized rats in doses sufficient to cause arrhythmias in control animals (Hejtmancik 1979). Ouabain caused a significantly greater decrease in heart rate in Pb-exposed than control animals, but no discernible differences in blood pressure increases, ECG waveform changes, or dosage required to produce arrhythmias was observed. It might be noted that ouabain causes bradycardia in part by an action mediated via the vagus nerve. Procainamide caused significantly greater decreases in heart rate and arterial pressure in Pb-exposed than in control animals. Twenty percent of control animals, but sixty percent of Pb-exposed rats, showed some degree of A-V block during or after procainamide infusion. The observation that drugs other than adrenergic agonists have greater effects in Pb-exposed than control animals suggests that some more general process than autonomic nerve function may be involved in the effect of Pb on the heart. One such possibility is an interaction of Pb with the processes controlling calcium movement.

LEAD AND CALCIUM

An interaction between Pb and calcium has been suggested by a number of studies on different model systems. Pb has been shown to cause decreases in serum Ca^{2+} (Kosmider et al. 1963). The administration of Pb decreases the uptake of ^{45}Ca into all brain areas (Burton, Johanson 1982). Kopp and Barany (1980) have demonstrated an antagonism between Ca^{2+} and Pb on cardiac contractility and myofibrillar protein phosphorylation. The negative inotropic effect and much of the ECG abnormality caused by Pb can be explained by interference with Ca^{2+} availability and/or membrane translocation.

CONCLUSIONS

The exposure to low concentrations of Pb during the neonatal period caused an enhanced responsiveness to the arrhythmogenic effect of NE, as well as an increased response to other cardioactive agents. A part of the effect seems to result from altered parasympathetic nerve activity and may be caused by changes in central autonomic control mechanisms. Evidence also indicates that Pb exposure may change the way the heart itself responds to cardioactive drugs, perhaps in part through alterations in adrenergic neuroeffector complex development, and in part by influencing the way in which Ca^{2+} is processed.

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THE CARDIAC-SYMPATHETIC AXIS AS A TERATOLOGICAL MODEL

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INTRODUCTION

The nervous system plays a vital role in the maintenance of cardiovascular function in the mature animal. In general, control is exerted through reflexes that operate in the central nervous system (CNS) and that adjust factors such as cardiac rhythm and contractility and vascular tone via efferent autonomic neurons, particularly sympathetic neurons. During perinatal development, sympathetic innervation of cardiovascular structures is immature or incomplete and becomes capable of controlling the circulation only gradually; in many species, including man, mature cardiovascular control does not appear until well after birth (Gootman *et al*. 1979; Slotkin *et al*. 1980). Before central control of the circulatory system is established, the neonate goes through a period in which various components of sympathetic axes are imprinted with the necessary information to ensure that future cardiovascular activity is appropriately regulated. While such programming is going on, the organism may be particularly vulnerable to agents that alter the development and/or expression of such control mechanisms.

An overall strategy for identifying how such perturbations come about should involve a model system in which defined neurotransmitters and synaptic components can be examined to see 1) how biochemical and physiological events contribute to the development of neuronal control of cardiovascular function, and 2) how alterations in these events during critical developmental periods result in abnormal functional status. The cardiac-sympathetic axis of the rat provides an ideal system in which to evaluate teratological mechanisms that operate to alter cardiovascular function. At birth, the rat does not possess functional sympathetic control of the heart, which enables the events surrounding the onset of function to be examined in the postnatal period. In addition, the neurons participating in control of cardiac-sympathetic function are well-defined in terms of transmitter biochemistry, anatomy, histology, and physiology, and the afferent and central mechanisms operating to influence sympathetic tone are established. This paper reviews what is known about how the cardiac-sympathetic axis develops, and it presents a classification system by which the actions of potential functional teratogens can influence the development of the cardiac-sympathetic axis.

DEVELOPMENT OF SYMPATHETIC CONTROL OF CARDIAC FUNCTION

The peripheral sympathetic nervous system of the rat is nonfunctional at birth and develops over the first three weeks postnatally in distinct stages:

1) Target organ receptors for noradrenergic stimuli are present at or before birth, yet responses to centrally mediated reflexes or to ganglionic stimulation are absent in the neonate (Adolf 1971; Bartolome _et_ _al_. 1977; Bareis, Slotkin 1978; Seidler, Slotkin 1979; Seidler, Slotkin 1981).

2) By the end of the first week postpartum, efferent sympathetic function becomes established, as a consequence of synaptogenesis at both ganglionic and postganglionic sites. In the cardiac-sympathetic axis, this is evidenced by onset of end-organ sympathetic responses to agents acting at these sites or via centrally mediated reflexes (Bareis, Slotkin 1978; Bareis, Slotkin 1980; Smith _et_ _al_. 1981).

3) Cardiac-sympathetic responses to baroreceptor afferent input do not appear until the second to third week postnatally; this final step leads to tonic sympathetic control of heart rate and is accompanied initially by a period of transient

sympathetic hyperactivity (Bartolome et al. 1980; Seidler, Slotkin 1981; Smith et al. 1982).

Evidence obtained in our laboratory indicates that each of these physiological stages is keyed to distinct biochemical and morphological events that occur in the development of neurons and synapses. The establishment of efferent sympathetic function awaits the arrival of noradrenergic synaptic vesicles in the postganglionic nerve terminals, as evidenced by the ability of isolated vesicles to take up and store [^{3}H]norepinephrine and by quantitative morphological estimations of vesicle packing density in axon bundles and nerve terminals (Bareis, Slotkin 1980; Slotkin et al. 1980). The onset of physiological function of noradrenergic synapses in the myocardium (evaluated by chronotropic responses to tyramine) correlates highly with vesicular uptake capacity, as does the onset of postganglionic neuronal function (tested by cardiac biochemical responses to nicotine) and overall function of the efferent sympathetic pathway (tested by centrally evoked reflex responses to hypoglycemia). To confirm that vesicle arrival is a major determinant of function, drug or hormone treatments that had been found to accelerate the onset of sympathetic function were examined; in each case, the agents first increased the rate of development of noradrenergic synaptic vesicles (Bareis, Slotkin 1980; Lau, Slotkin 1980; Slotkin et al. 1980).

Although both the biochemistry and the physiology of the efferent sympathetic pathway to the heart indicate that functional neurotransmission can occur by one week postnatally, the sympathetic system does not participate in tonic control of heart rate until the second to third week. Thus, administration of ganglionic blocking agents, which ordinarily cause a reduction in heart rate in the mature animal, did not do so in the one-week-old neonatal rat (Seidler, Slotkin 1979). The developmental step that appeared to be lacking was the central processing of baroreceptor input, which controls sympathetic output; one-week-old rats did not display cardioacceleration or cardiac biochemical responses when hypotensive drugs were administered, although agents that evoke ganglionic or centrally evoked sympathetic stimulation were fully effective (Bartolome et al. 1980). Similarly, hypotension could not evoke an increase in norepinephrine turnover in cardiac sympathetic nerves until the second to third postnatal week, even though efferent sympathetic function was present by the end of the first week (Seidler, Slotkin 1981). After the establishment of baroreceptor control of sympathetic output, there was a transient period of sympathetic hyperactivity during the third week, as evidenced by increased norepinephrine turnover and an elevation in the nerve-impulse activity recorded from preganglionic sympathetic trunks (Seidler, Slotkin 1981; Smith et al. 1982).

During postnatal development, then, the rat goes through a period in which cardiac control by the CNS is largely absent, followed by a stage in which responses to some acute stressors are possible, and finally a stage in which complex reflexes (such as those mediated through baroreceptors) become evident. With these factors in mind, it is possible to typify how teratological alterations in cardiac-sympathetic function can come about. The next section of this paper summarizes the different types of abnormalities that have been reported.

TERATOLOGY OF CARDIAC-SYMPATHETIC FUNCTION

The clear division of development of the cardiac-sympathetic axis into different maturational phases enables a classification of types of alterations in function that can be elicited by perinatal insults. First, and most obviously, any exposure that alters the development of sympathetic synapses will alter the capability of the CNS to control cardiac function. Agents that cause this type of alteration would be expected to influence the time of onset of synaptic function in the sympathetic nervous system and/or to affect function of specific presynaptic or postsynaptic elements participating in synaptic transmission (neurotransmitter synthesis, storage, or release, and receptor number or sensitivity). The timing of the appearance of these defects should correspond to the period in which synaptogenesis is occurring in the efferent sympathetic pathway, i.e. in the first 10 days postnatally. The second type of alteration is caused by factors that affect CNS areas involved in autonomic function and exert a more subtle effect on the cardiovascular system through abnormalities in sympathetic tone. The abnormalities are not necessarily accompanied by morphologically or biochemically detectable changes in peripheral sympathetic synapses per se. These types of perturbations appear only after the period in which the CNS becomes capable of regulating sympathetic tone.

TREATMENTS AFFECTING DEVELOPMENT OF SYMPATHETIC SYNAPSES

A wide variety of agents can cause alterations in development of synapses in the cardiac-sympathetic axis. Perinatal exposure to ethanol, opiates, hyperthyroidism, or methylmercury all have been found to accelerate the onset of synaptic function (Bareis, Slotkin 1978; Bareis, Slotkin 1980; Lau, Slotkin 1979; Lau, Slotkin 1980; Bartolome *et al*. 1981; Bartolome *et al*. 1983); in each case, the alterations appear to involve precocious maturation of the presynaptic noradrenergic nerve terminals in the myocardium. The question then arises as to whether such premature functional capabilities are at all deleterious to the developing organism. In fact, substantial evidence has been obtained that suggests that the early appearance of sympathetic input to cardiac tissue produces a long-term disability of neuronal activity as well as adversely affecting development of the myocardium itself. The sequence of events participating in the eventual deficits has been best typified for the case of neonatal hyperthyroidism (Lau, Slotkin 1979; Lau, Slotkin 1980; Smith *et al*. 1981). Within 24 h after administration of thyroid hormone to a one-day-old rat, the cardiac-sympathetic axis becomes capable of transmitting nerve signals across ganglionic and postganglionic synapses; this capability thus appears nearly a week earlier than it would have normally.

Thyroid hormone enhances the maturation of noradrenergic nerve terminals in the myocardium, but at the very same time it causes a drastic reduction in the number of growth cones associated with developing axonal projections (unpublished observations). Consequently, the premature onset of function is associated with a later deficit in the number of nerve terminals. In addition, thyroid hormone causes an early rise in the number of β-adrenergic receptors on myocardial cells, but the development of these receptors is then shut off prematurely, leading to a later deficit in end-organ neurotransmitter receptors. The presynaptic and postsynaptic deficits combine to produce eventual subsensitivity of the myocardium to sympathetic stimulation and thus a blunting of functional response, which first appears during the third postnatal week and continues into adulthood.

These results indicate that exposures to agents that evoke premature onset of sympathetic synaptic function are certainly not beneficial to the organism, and are not even innocuous. Rather, the precocious appearance of sympathetic capability produces an eventual deficit as the animal matures. Similar findings have appeared for the other treatments that accelerate sympathetic synaptogenesis; indeed, even relatively minor maternal stress at near-term may be capable of eliciting such alterations with long-term consequences for sympathetic regulation of blood pressure and heart rate (Smith, Mills 1983).

Just as there are agents that accelerate the onset of sympathetic function, there are treatments that slow down or eliminate the appearance of neurotransmission. These include neonatal exposure to drugs that deplete catecholamines from sympathetic neurons (reserpine [Bartolome *et al*. 1977]), neurotoxins that destroy sympathetic nerve terminals (high doses of guanethidine [Bareis *et al*. 1981]), hormones that uncouple cardiac β-adrenergic receptors from physiological response of cardiac muscle (glucocorticoids [Lau, Slotkin 1981]), or hormone deficiencies that retard development of presynaptic and postsynaptic components of noradrenergic terminal areas (hypothyroidism [Lau, Slotkin 1982]). Although the specific synaptic site of action of each of these compounds differs, the net physiological result of each is similar in that the capability of sympathetic nerves to elicit cardioacceleration appears later than it should. In each of these cases, there is an additional effect of slowing cardiac growth, sometimes to the extent that the heart weighs 40% less than normal hearts. While some of the retardation of cardiac growth undoubtedly reflects direct effects of the perturbing agents on the myocardium, it is also possible that the alterations in sympathetic function participate directly in such actions.

The release of catecholamines from sympathetic neurons is thought to play a key role in development of myocardial cells. β-Adrenergic stimulation terminates the phase of DNA replication in immature cardiac muscle and initiates the phase in which further growth occurs primarily by RNA-dependent hypertrophy of preexisting cells (Claycomb 1976). It would thus be expected that agents that retard the development of sympathetic synaptic function would also influence adversely the growth patterns of the myocardium. Interestingly, this hypothesis would predict that the opposite case, i.e. accelerated onset of sympathetic function, would also produce eventual retardation of cardiac growth as a consequence of premature termination of DNA replication. This effect has been observed (Bareis, Slotkin 1978; Bareis, Slotkin 1980; Lau, Slotkin 1979; Lau, Slotkin 1980). Thus, the effects of perinatal insults on neuronal components of the cardiac-sympathetic axis can also influence development of heart tissue.

TREATMENTS AFFECTING CENTRAL REGULATION OF SYMPATHETIC TONE

In addition to teratological alterations in which sympathetic neuron synaptogenesis is directly affected, loci in the immature CNS that control sympathetic tone are also sensitive to perturbation during development. In cases where central alterations are present, sympathetic neurons typically develop in proper fashion up until the time when central control of sympathetic tone is first exerted, in the second to third postnatal week. At that point, the centrally mediated abnormalities in sympathetic tone are expressed as corresponding changes in function of peripheral synapses and end-organ activity. Thus, drug- or toxin-induced alterations in development of the cardiac-sympathetic axis can involve sites remote from the heart or sympathetic neurons.

Prime examples of centrally mediated functional teratology are provided by the action of agents that influence biogenic amine systems in the developing brain during the critical periods in which centers controlling cardiovascular reflexes are being established. Administration of 6-hydroxydopamine directly into the CNS of neonatal rats destroys catecholaminergic nerve terminals selectively, without directly influencing synaptogenesis in peripheral neurons (Deskin *et al*. 1980); animals lesioned in this fashion develop quite normally initially. Subsequently, when tonic control of sympathetic activity appears, the affected animals do not show the typical increase in sympathetic tone that normally occurs in the third postnatal week, and as they grow into adulthood they display abnormally low tonic sympathetic activity and high parasympathetic activity (Deskin *et al*. 1980; Deskin, Slotkin 1981). Because of the participation of catecholamines in cardiac growth, the depressed sympathetic tone leads to deficits in heart weight that appear in the third postnatal week and become quite substantial during growth into adulthood. The neuronal and cardiac alterations result in a low resting heart rate despite normal blood pressure, suggesting that cardiac output is depressed. These animals are also incapable of withstanding stressors that require long-term elevations of sympathetic tone, such as salt-load.

Administration of another biogenic amine depleting agent, reserpine, to pregnant rats in mid-gestation produces an opposite type of reaction: the offspring develop a permanent elevation of sympathetic tone (Bartolome et al. 1976; Lau et al. 1977). Again, the central origin of this is apparent from the time course with which the alteration appears. Indices of sympathetic activity and synaptogenesis appear normal in the initial postnatal period, but activity becomes permanently elevated once the CNS becomes functionally connected to sympathetic efferents. Again, these animals are unable to "turn on" sympathetic activity appropriately during stress (Dailey 1978). The peripheral neuronal changes are preceded by metabolic changes in central neurons containing transmitters thought to participate in sympathetic-cardiovascular regulation (Lau et al. 1977). It is apparent, then, that functional teratology of this type displays the same "critical period" requirements that are usually associated with classical morphological teratology. Reserpine, which depletes catecholamines and other biogenic amines, exerts its promoting effect on sympathetic tone only when fetuses are exposed during a distinct three-day period in gestation; destruction of these same nerve terminals later in life (neonates given 6-hydroxydopamine) produces a very different (in fact, opposite) effect.

Centrally mediated functional alterations in sympathetic regulation of the cardiovascular system are not limited to agents that affect catecholamines. Depletion of histamine, another central biogenic amine transmitter thought to participate in cardiac-sympathetic regulation, also leads to teratologic alterations in sympathetic tone that result from a centrally derived mechanism (Slotkin et al. 1983). In this case, administration of alpha-fluoromethylhistidine, a specific inhibitor of histamine synthesis, produced a large, target-specific increase in turnover of catecholamines in sympathetic nerves, again derived from primary effects on the CNS. As would be expected, the turnover alterations occur only after the CNS is capable of regulating sympathetic tone, i.e. in the second to third postnatal week. The increase in transmitter turnover leads to corresponding deficits in norepinephrine content in the developing myocardium; the functional correlates of this type of defect are under investigation.

CONCLUSIONS

The cardiac-sympathetic axis provides a useful model with which to examine how perinatal insults influence neuronal cardiovascular function as well as cardiac growth. Because the mechanisms operating in the development of this system are easily identifiable, it is possible to pinpoint the site of origin of teratological alterations resulting from exposure to drugs, toxic chemicals, hormonal imbalances, or maternal stress. Adverse effects fall into two broad categories: 1) insults that directly influence synaptogenesis and function of peripheral sympathetic neurons, and 2) those that exert more subtle effects on CNS regulation of sympathetic activity. Some of the deleterious effects seen in development of cardiac tissue may have their origin in alterations in the development, function, or central regulation of cardiac sympathetic pathways.

ACKNOWLEDGMENTS

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DISCUSSION SUMMARIES--HEART

CARDIOVASCULAR PROBLEMS OF THE NEWBORN AND THEIR ETIOLOGIES
(Herbert S. Harned, Jr.)

DR. KIMMEL: You mentioned that salicylates might be a problem. Can you tell me what information supports that assertion?

DR. HARNED: Salicylates are teratogenic in animals. Aspirin and other prostaglandin synthesis inhibitors are very reactive in fetal animals in closing the ductus, which is a potentially devastating situation. Many people are also worried about the bleeding that could be caused by salicylates, and I don't know if this is related to some prematurity or not.

DR. BALLARD: As I recall David Smith's work on fetal alcohol syndrome, a single binge of heavy drinking by the mother could produce the syndrome. Can a single episode of heavy drinking produce cardiac abnormalities?

DR. HARNED: Usually we see the fetal alcohol syndrome with cardiac manifestations in a flagrantly alcoholic female. I don't recall seeing the cardiac conditions resulting from a single binge.

DR. GRABOWSKI: It's controversial whether a single binge can do it.

DR. GROSS: About eight years ago, no one ever heard of persistent pulmonary hypertension and now it obsesses people. Do you think we've just become aware of it, or do you think its incidence has changed?

DR. HARNED: I can only speculate, but I don't think this is a new syndrome. We used to occasionally find children with pulmonary hypertension that we've actually carried to the point of cardiac catheterization. With more babies coming into tertiary centers, conditions like that are going to be recognized more. What's your feeling about that?

DR. GROSS: I think it's probably a matter of recognition.

DR. HARNED: The syndrome is difficult to treat. We still lose a lot of these babies.

THE ELECTROCARDIOGRAM OF FETAL AND NEWBORN RATS AND DYSRHYTHMIAS INDUCED BY TOXIC EXPOSURE

(Casimer T. Grabowski)

DR. HARNED: Do you think the edema is secondary to heart failure?

DR. GRABOWSKI: I don't know. In earlier studies with moderate doses, the edema is so conspicuous that I think it's a primary effect. But with low doses, the edema is not readily apparent.

DR. SLOTKIN: The greater sensitivity in the immediate prenatal period suggests to me that you might have an EKG defect that's generated by something having to do with the development of autonomic innervation of the heart. The period just surrounding birth is when the vagal innervation can transmit signals to the heart, while sympathetic innervation in the rat cannot. If you turned on the vagus prematurely or made the vagus develop synapsis a little earlier, you could conceivably produce heart block. If that's the case, then you should be able to resolve the heart block with atropine. Have you tried that?

DR. GRABOWSKI: No, but that is one of the things I have in progress.

DR. KAVLOCK: I was glad to see your positive control data because that's an issue we're often facing in a regulatory agency. Often the effects that are seen are attributed to concurrent maternal toxicity. It's good to see that you can induce maternal toxicity and not get any functional defects. We have data from several pesticides where we get a lot of maternal toxicity and mortality and almost zero weight gain during gestation, yet we see fetuses that look healthy and well.

DR. GRABOWSKI: There are a lot of rats in that series, and I think it's a valid positive control.

DR. WILLIAMS: Have you allowed your mirex-treated animals to mature and looked at them as adults?

DR. GRABOWSKI: I don't have enough facilities to do this routinely. I think they should be kept and then stress tested in some way. The usefulness of such testing has been frequently demonstrated during this conference.

ELECTROCARDIOGRAPHIC STUDIES OF DEVELOPING RODENTS EXPOSED _IN UTERO_ TO TRYPAN BLUE (William P. Watkinson _et al_.)

DR. SMITH: I believe you said that the incidence of individual fetuses with EKG irregularities does not appear to be dose related, but the number of irregularities within those individuals was dose related. With this toxin, or with others, are there different types of individuals in the population who are susceptible and some who aren't, and does that raise any wider regulatory questions? Are there any examples where one might search out the susceptible portion of the population and seek to protect them rather than apply it to general populations?

DR. CHERNOFF: Any regulatory agency does target sensitive populations, but that target is dependent upon the size and often the vocalness of the sensitive population. For instance, asthmatics and similar populations are very carefully studied, and part of the reason for this conference lies with the target population of children. So yes, that is a regulatory concern and there is a chance you are dealing with a subset of your population that is sensitive.

DR. SMITH: What then is the regulatory response? Is it to provide population-wide standards low enough to protect the most sensitive individuals, or for instance, to move somebody from Dade County to North Overshoe County?

DR. CHERNOFF: It depends on the size of the population. There's no scientific answer to that. It's strictly a political answer.

DR. HARNED: There is a striking difference between these two papers. The damage to Dr. Grabowski's animal is toward the atrium and AV conduction bundle. What I'm seeing in your animal is heart muscle damage. Did you find heart muscle changes in the pups that you autopsied?

DR. WATKINSON: We didn't look at that.

DR. SPITZER: Is trypan blue used in any other instances?

DR. WATKINSON: I don't really know. It's used primarily in teratology studies because it's a known teratogen.

DR. GRAY: It's an interesting compound and many attempts have been made to identify its mechanism of action. It's interesting in that none of it reaches the fetus, but it gets to the yolk sac early in gestation.

DR. GRABOWSKI: I've experimented with trypan blue from that standpoint. Part of the problem is that it's a mixture of dyes so the blue part may not be reaching the fetus but some other dye may be. It produces a lot of fluid imbalance in the fetuses and they swell. At that time the heart is twisting and turning, and I think that is responsible for some of these transpositions.

DR. SPITZER: Why do they swell?

DR. GRABOWSKI: That's what I would like to find out, but it does seem to affect the yolk sac placenta, which is the functional placenta at these early stages. Later, trypan blue is not effective at all when the yolk sac placenta decreases in importance. You have to get it in the early stages. It's an anomaly, but the dye has been widely used because it was useful in producing a lot of heart malformations.

CARDIOTOXICITY OF PERINATAL LEAD EXPOSURE

(Betty J. Williams and Mary E. Abrue)

DR. SLOTKIN: The antagonist vs. agonist forms of the beta receptor could be studied. The coupling ratio of beta receptors in the heart is so variable with drug treatments, toxins, or development that it's likely you can have something that's biochemically subtle, but that could have tremendous impact on myocardial irritability to catecholamines.

DR. WILLIAMS: That's very true.

DR. SPITZER: Dr. Williams, the emphasis of your presentation has been cardiac effects. You haven't made too many comments on blood pressure. I notice that you have a slight difference in blood pressure compared with nonexposed animals. Did you follow these animals?

DR. WILLIAMS: We've never seen any statistically significant differences. The lead-exposed animals tend to be a little hypertensive, but we've never seen any frank hypertensive animals relative to controls.

DR. GRABOWSKI: When the DES story came out, with delayed effects occurring many years later, people wondered what other less common delayed effects were possible. During this conference, we are seeing a lot of functional effects that don't appear until some time after birth, so I think that this is a real concern.

DR. SPITZER: To add to that, we are always hearing, particularly in Australia, that a large population of children have been exposed to lead early in life, and they've had evidence of renal deficiency 30 or 40 years after the lead exposure ceased.

THE CARDIAC-SYMPATHETIC AXIS AS A TERATOLOGICAL MODEL (Theodore A. Slotkin)

DR. GRABOWSKI: Your studies have demonstrated a functional equivalent to prenatal exposure to DES, a long-term delay in the toxic reaction. Whenever you get a difference in delay of reaction for different individuals, we immediately say that these are genetic differences in the population. Here is a basis for many differences in reaction to something (drugs, stresses, or whatever) that are clearly not genetic.

DR. SLOTKIN: I agree. It's puzzling that, for some of these agents, particularly the opiates and alcohol, we get a separation of the populations: one is developing normally and the other is really badly affected. Here you've got genetically matched animals and they're not responding the same way.

DR. SMITH: At the European pediatric meetings, there was an interesting abstract suggesting nongenetic transgenerational transmission of disease and maternal diabetes leading to fetal pancreatic hyperfunction. Then that fetus grew up, exhausted early, became a diabetic, and the condition perpetuated itself. With this kind of maternal stress, is it possible that this could be a transgenerational effect?

DR. SLOTKIN: Yes, and it could be mistaken for a genetic effect. A good example is maternal reserpine treatment when reserpine was the drug of choice in pregnant women with toxemia. In our animal studies, we see the rats turning on their sympathetics permanently, which is at least a putative cause of essential hypertension, so a nongenetic transmission of essential hypertension is possible.

DR. DASTON: It's very interesting that corticosteroids and T_3, used in clinical settings to save premature infants from respiratory distress syndrome, would adversely affect the sympathetic nervous system. This seems to be an ideal population of humans for studying adverse development of the sympathetic nervous system as based on your results.

DR. LANGSTON: There are tremendous timing differences between the rat and the human. The change from cell growth or cell division to increase in cell size happens before mid-gestation in the human, so the effects of hormones influencing the systems don't happen at the period they're being treated (near the end of gestation).

DR. GROSS: I think in comparing animals to man you have to consider two things: first, the dose, and second, relative timing of organ maturation. Because steroids cause an adverse effect in the development of the brain in rats, there's considerable concern that it may do the same in humans. Very careful and thorough developmental product studies as well as neurological studies have now been done on infants who received corticosteroids, and the data indicate no adverse effects on either neurological or behavioral outcome at about five years of age.

DR. SLOTKIN: The problem here is that the changes we see are sufficiently subtle in some cases that a neurological test may not pick them up. It's a question of what you're looking for. Since we don't know the connection between a specific synapse or change in receptor and behavior, we may not be making the right behavioral test. It would be nice to do long-term follow-up studies. Maybe five years isn't enough. The same thing was thought with women and their offspring in the methadone program: that neurological deficits resolved within a few years. The latest studies indicate that when these children get to be eight and ten years old, they start to have learning deficits, so it's something that has to be monitored over a very long time period, and the question of which tests are appropriate and how these tests could be matched with biochemical changes that can be picked up in the rat is difficult to answer at present.

DR. SMITH: Following up on Dr. Daston's comment, I think that we have been very concerned about using these kinds of agents, and I think we do have to keep our minds open to this possibility. In my mind, the tragedy of the DES story is not that it led to long-term teratology, but that it led to long-term toxic effects with no benefit. I think as long as we're doing something that has a defined benefit, we are more prepared to accept this long-term possibility.

NORMAL ORGANOGENESIS OF THE HUMAN KIDNEY

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INTRODUCTION

Before considering the embryogenesis of the human kidney, it is helpful to examine the final result of this elaborate program by examining the gross anatomy of the normal mature kidney. The mature kidney of man has a multi-lobulated formation and its overall architectonic design is uniquely suited to accomplish this goal. The vital importance of the spatial organization required for normal renal function is best appreciated in a longitudinal cross section showing individual nephron units. This is shown diagrammatically in Fig. 1.

Glomerulo-Nephron Structure

There are about a million glomerulo-nephron units in each kidney. Each nephron tubule consists of sequential segments that have different structure and different functions, and the location of each segment in a specific region of the kidney (i.e., cortex or medulla) is preordained to conform to the spatial orientation required for the complex processes whereby urine is formed from a protein-free ultrafiltrate of plasma. The first part is the renal corpuscle (glomerulus) (Fig. 1), consisting of a tuft of capillaries situated within Bowman's capsule. Not all glomeruli are of identical size. Those situated in the deeper layers of the cortex (juxtamedullary nephrons) are largest.

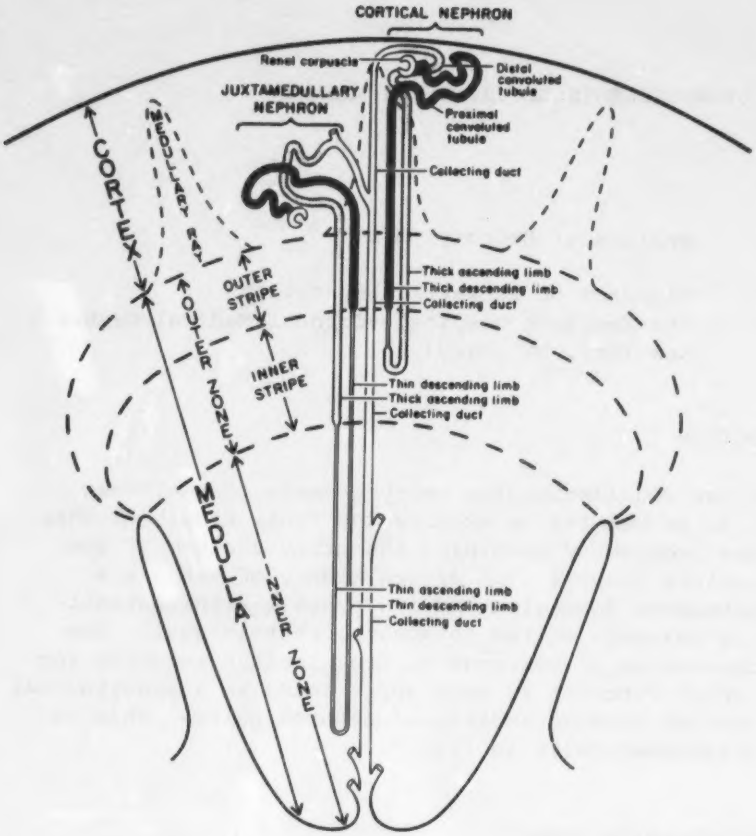
CORTICAL NEPHRON
Renal corpuscle
Distal convoluted tubule
JUXTAMEDULLARY NEPHRON
Proximal convoluted tubule
Collecting duct
CORTEX
MEDULLARY RAY
OUTER STRIPE
INNER STRIPE
OUTER ZONE
MEDULLA
INNER ZONE
Thick ascending limb
Thick descending limb
Collecting duct
Thin descending limb
Thick ascending limb
Collecting duct
Thin ascending limb
Thin descending limb
Collecting duct

Fig. 1. Schematic diagram of a cortical and a juxtamedullary nephron showing the relationship of nephron segments to zones of the kidney. [From: Strauss MB, Welt LG (1979). "Diseases of the Kidney," 3rd ed, Vol 1. Boston: Little, Brown and Company. Copyright 1979.]

Bowman's capsule, the epithelium-lined dilatation of the nephron, is invaginated by a unique filter formed by a tuft of capillaries supplied by an afferent arteriole and re-anastomosing to form an efferent arteriole. This permits a high hydraulic pressure filter to function in the glomerulus. There is a narrow, capsular space between the outer lining of Bowman's capsule (parietal layer of capsular epithelium) and the inner or visceral layer (glomerular epithelium) which closely invests the capillary tuft. The glomerulus has a vascular pole where afferent and efferent arterioles enter and leave, and a urinary pole at the opposite side of the corpuscle where capsular space is continuous with the lumen of its proximal convoluted tubule and where the parietal (squamous) epithelium is continuous with and now forms the cuboidal or low columnar epithelium of the proximal convoluted tubule.

Urinary Tubule

The urinary tubule of each renal corpuscle begins with the convoluted portion of the proximal tubule (Fig. 1). Next are the straight portion of the proximal tubule, the thick and thin descending limb segment, and the loop of Henle, which enters the medullary ray and loops into the medulla, returning to the cortex via the same medullary ray. As the distal tubule returns to the cortex, it passes by the renal corpuscle from which the nephron originated (Fig. 1), forming the macula densa of the juxtaglomerular apparatus and continuing as the distal convoluted tubule within the cortex. The lengths of the loops of Henle vary somewhat with the position of the renal corpuscle in the cortex. Nephrons in the outer region of the cortex (cortical nephrons -- the last to be formed) have short loops of Henle with short or nonexistent thin limb segments. Nephrons near the cortico-medullary junction (juxtamedullary nephrons -- 15-20% of the total and the first to be formed) have long loops of Henle with long descending and ascending thin limb segments (Fig. 1). These thin limbs run parallel with the collecting ducts in the outer and inner zones of the medulla. All of the distal nephrons empty into their collecting ducts in the cortex. These anatomical arrangements are of great significance to the complex functions involved in the normal processes whereby urine is diluted or concentrated.

EMBRYOGENESIS OF THE KIDNEY

Traditional embryological teaching presents a taxonomic description of the early stages wherein the major changes in gross appearance are used to separate renal organogenesis into three stages: pronephros, mesonephros, and metanephros (McCrory 1972).

The Pronephros

The first evidence of a renal excretory system in the human embryo consists of individual paired tubules that arise from nephrotomes that form a hollowed-out vesicular structure called the pronephric tubule. The first tubules appear about the middle of the 3rd week (8- to 9-somite embryo). Each proliferating tubular bud grows caudally until it fuses with the tubular bud of the next pronephric tubule produced, giving rise to a most important structure, the pronephric duct, which persists after the pronephric tubules regress (Fig. 2A). The pronephros is of no significance as a functioning excretory organ. It is important because it gives rise to the mesonephric duct, which produces one of the unique tissues (the ureteric bud) required for the formation of the metanephros.

The Mesonephros

The mesonephros appears as a more complex structure immediately caudal to the last of the pronephric tubules (Fig. 2A). Vesicles now appear within the nephrogenic cords, induced by interaction of the pronephric duct (now the mesonephric duct) with the nephrogenic mesenchyme forming the nephrongenic cords. The mesonephros appears at about the 3rd week. As with the pronephros, the earliest nephrons formed (cephalic) begin to degenerate (5th week), while the more caudal nephrons are still differentiating. The "organ" thus shifts caudally by this process. The majority of mesonephric nephrons have degenerated by the 11-12th week.

The mesonephric nephrons are the first true glomerulotubular units. The individual vesicles differentiate into a primitive S-shaped tubule whose distal projections establish continuity with the mesonephric duct. The proximal ends broaden and form a hemispheric two-layered cup, and capillaries vascularize this primitive Bowman's capsule. The tubules differentiate into a definitive single tubule with convolutions and a brush border, but they have no loop of Henle or distal tubule. In renal ontogeny, these

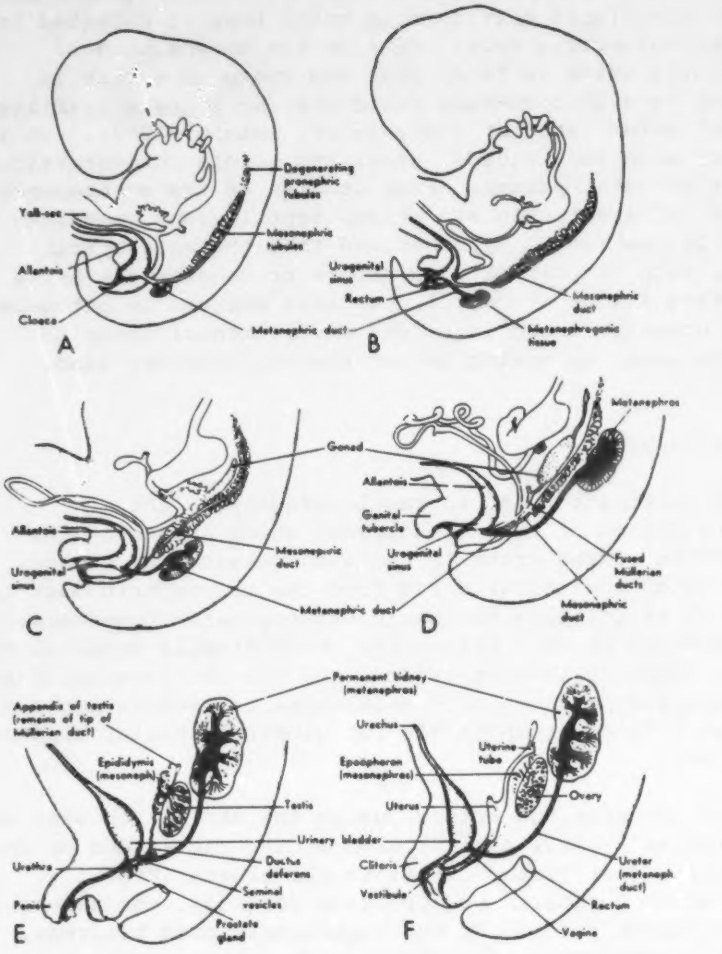
Degenerating pronephric tubules
Yolk-sac
Mesonephric tubules
Allantois
Cloaca
Metanephric duct
A
Urogenital sinus
Rectum
Mesonephric duct
Metanephrogenic tissue
B
Gonad
Allantois
Mesonephric duct
Urogenital sinus
Metanephric duct
C
Metanephros
Allantois
Genital tubercle
Urogenital sinus
Fused Müllerian ducts
Mesonephric duct
D
Permanent kidney (metanephros)
Appendix of testis (remains of tip of Müllerian duct)
Epididymis (mesoneph)
Testis
Urinary bladder
Urethra
Ductus deferens
Seminal vesicles
Penis
Prostate gland
E
Urachus
Uterine tube
Epoöphoron (mesonephros)
Ovary
Uterus
Clitoris
Vestibule
Ureter (metaneph duct)
Rectum
Vagina
F

Fig. 2. Relative size and positions of nephric organs of the human embryo at various stages of development. A. Early in fifth week (adapted from several sources covering 5-6 mm embryos). B. Early in sixth week (modified from Shikinami's 8 mm embryo). C. Seventh week (modified from Shikinami's 14.6 mm embryo). D. Eighth week (adapted from Shikinami's 23 mm embryo and the Kelly and Burnam 25 mm stage). E. Male at about 3 months, schematized. F. Female at about 3 months, schematized. [Reproduced with permission from: Patten BM (1968). "Human Embryology," 3rd ed. New York: McGraw-Hill Book Company. Copyright 1968.]

tubules collectively constitute the first true glomerular tubular functional unit forming urine that is excreted into a common collecting duct. Data on the composition of mesonephric urine in fetal pigs and sheep show that it consists of a glucose-free fluid similar to an ultrafiltrate of fetal serum (Stanier 1960; Perry, Stanier 1962). It is isomolar with body fluids, urea, and solute concentrations similar to fetal plasma. From studies of the mesonephros in a number of anamniotes and avian, reptile, and mammalian forms, Davies (1952) hypothesized that the metanephric kidney, with its capacity to dilute or concentrate urine (requiring a loop of Henle), probably emerged in ontogenesis as the organism faced increased environmental needs to conserve water by moving out of the sea onto dry land.

The Metanephros

The ultimate event in renal ontogeny is the differentiation of the metanephros, which arises by the interaction of the ureteric bud with mesenchymal tissue. Induction of the ureteric bud from the mesonephric duct is essential to initiate two basic organogenetic processes: the induction of duct branching, which finally produces the whole urinary collecting system, and the interaction of the duct ampullae produced with mesenchyme to produce nephronic vesicles that are responsible for glomerulotubular nephron formation.

The ureteric bud arises during the 4th to 5th week of gestation as a small diverticulum at the caudal end of the mesonephric duct just proximal to the cloaca (Fig. 2A). Growth of the ureteric bud proceeds dorsally, encountering the caudalmost portion of the nephrogenic cord blastema, which then condenses and surrounds the growing ureteral bud as a mass of cells that will from the metanephrogenic tissues.

The ureteric bud begins to divide at about the 6th week. The first few bud divisions are not associated with induction of nephrons (Fig. 3). They first appear on the 2nd to 5th generation of ureteral bud branches at about 8 weeks.

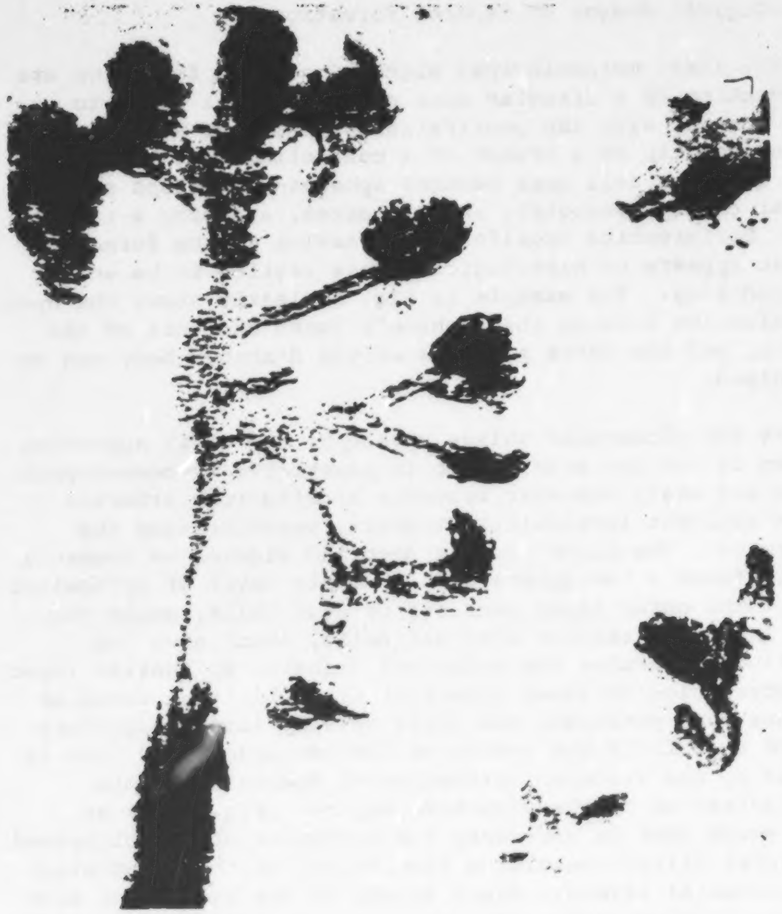

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Fig. 3. Microdissection of left kidney and ureter from 18 mm, 8 week embryo. Ureteral bud has divided 4-6 times in polar area and 3-4 times in interpolar area. All branches present at this stage will be expanded into renal pelvis and calyces during subsequent development. Renal vesicles desired from nephrogenic blastema are first present in relation to ampullary ends of 3rd-5th generation of ureteral bud branches. [From: Potter EL (1972). "Normal and Abnormal Development of the Kidney." Chicago: Year Book Medical Publishers.]

Morphological Stages of Nephron Formation

The first morphological signs of nephron formation are condensation of a circular mass of mesenchymal cells in close contact with the proliferating tissue of ampulla forming the tip of a branch of a collecting-duct tubule (Fig. 4). The cell mass becomes spheroid, develops a central cavity (vesicle), and elongates, assuming a tubular form. Differential proliferation results in the formation of what appears on histological cross section to be an S-shaped body. The example in Fig. 5 clearly shows the open communication between the nephron's lumen and that of the ampulla, and the three portions of the S-shaped body can be recognized.

As the glomerular anlage develops, the cleft appearing between it and the middle limb is penetrated by mesenchymal tissue and small vascular branches arising from arteries within adjacent interstitial tissues, vascularizing the glomerulus. The cleft becomes deep and widened as Bowman's capsule forms a hemisphere with a double layer of epithelial cells. The outer layer consists of flat cells, while the inner layer has thicker cuboidal cells, which give the primitive glomerulus the prominent visceral epithelial layer characteristics of fetal glomeruli (Fig. 5). The vascular branches that penetrate the cleft develop into a capillary network that fills the cavity of the hemisphere and that is covered by the visceral epithelium of Bowman's capsule. Vascularization of the primitive nephron (Fig. 6) is an early event that is necessary for formation of the bilayered glomerular filter, resulting from fusion of the endothelial and epithelial layers. Rapid growth of the glomerulus with its capillary network pushes the adjacent middle limb of the S-shaped body (Stoerk's complex) away, resulting in early positioning of the distal limb of Stoerk's complex (responding to the future distal tubule and macula densa of the juxtaglomerular apparatus) in close proximity to the vascular pole of the glomerulus (Fig. 6). This juxtaposition will be retained throughout the future nephronic development. The upper edge of Bowman's capsule constricts, bringing the efferent and afferent vessels together at the vascular pole of the glomerulus opposite the exit of the proximal tubule. The developing nephron thus establishes two fixed physical attachments very early, one to its branch of the collecting duct and the other with the

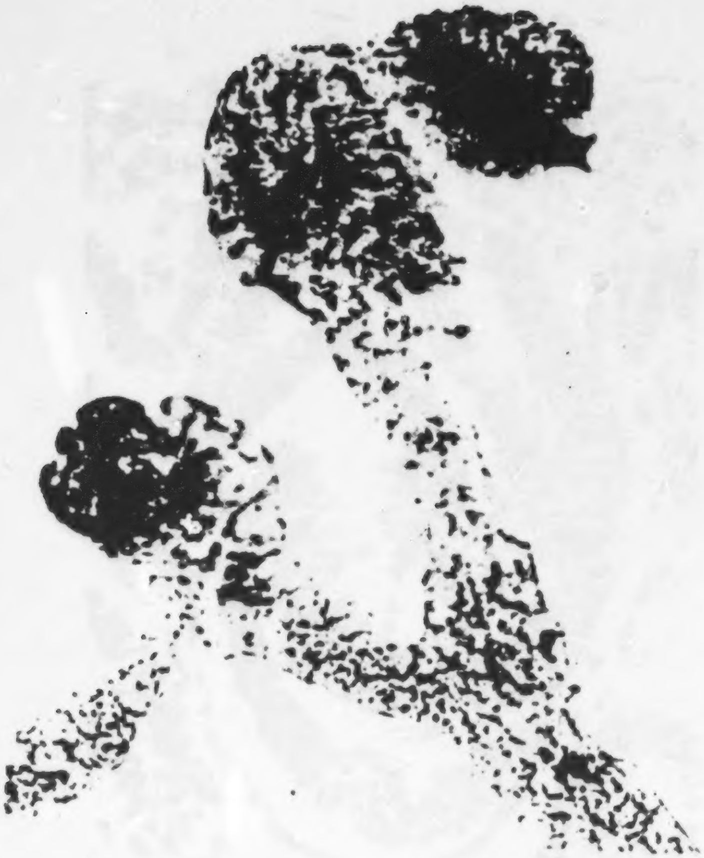

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Fig. 4. Earliest evidence of vesicle induction. A divided collecting tubule ends in two ampullae. At the lower left, a vesicle is seen as a disc-like mass of deeply stained nephrogenic mesenchyme. Attached below is the connecting tubule of a previously induced nephron. Above and to the right is an ampulla with a fully developed spherical vesicle in which a central cavity can be dimly seen. [From: Oliver J (1968). "Nephrons and Kidneys: A Quantitative Study of the Developmental and Evolutionary Mammalian Renal Architectonics." New York: Hoeber Medical Division, Harper and Row.]

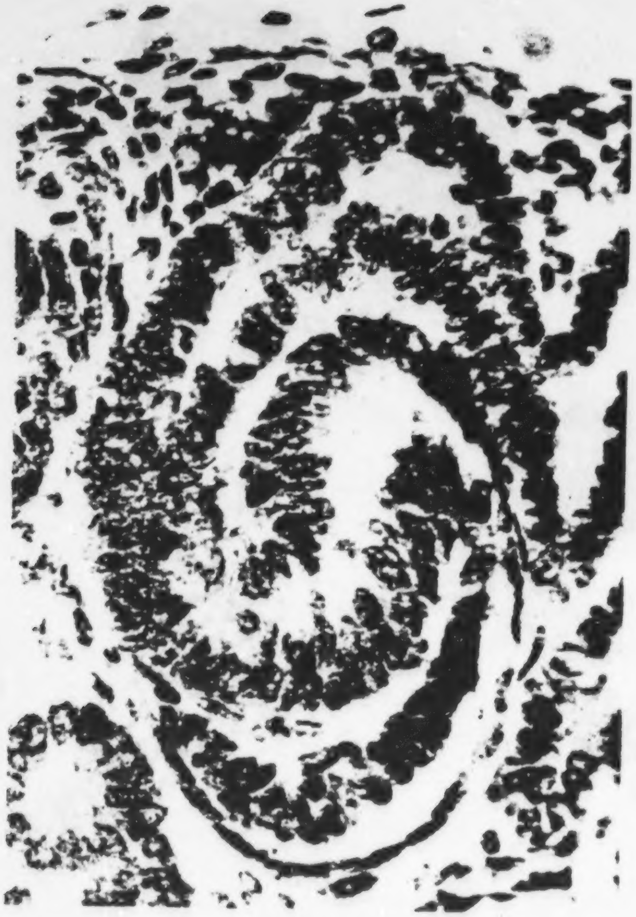

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Fig. 5. Nephron in S-stage attached to ampulla before there is appreciable lengthening of the collecting tubule. Open communication between nephron's lumen and that of ampulla is evident. Three portions of this S-shaped body can be recognized: 1) the upper limb continuous with the collecting tubule (top of Fig. 4), 2) the middle limb (Stoerk's complex) which will form the proximal tubule and loop of Henle, and 3) the lower limb, which already has formed a space between the visceral and parietal epithelial layers, with a cleft evident between it and the middle limb (bottom of Fig. 4). [From: Potter EL (1972). "Normal and Abnormal Development of the Kidney." Chicago: Year Book Medical Publishers.]

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Fig. 6. Vascularization of the rudimentary glomerulus. The dislocation of the two coils of Stoerk's complex from the glomerular cup has occurred spontaneously. The edges of the cup have extended to form an avoid hemisphere into the cavity of which a thick afferent vessel extends. The proximity the arterial vessels would have to the first portion of the distal tubule before dissection is evident. [From: Oliver J (1968). "Nephrons and Kidneys: A Quantititative Study of the Development and Evolutionary Mammalian Renal Architectonics." New York: Hoeber Medical Division, Harper and Row.]

vascular pole of the glomerulus and the distal tubule at the site of the future macula densa.

Formation of the Pelvicalyceal System

The earliest ureteric bud branches dilate to form the primitive renal pelvis and major calyces, which can be seen by 9 weeks. Since this dilation is present with functioning glomeruli (Fig. 7), it seems likely that its expansion is related to the accumulation of urine secreted by the fetal kidney. The pelvis and calyceal system expand and can be clearly demarcated from the profusion of terminal branching tubules with clusters of attached nephrons by 11 weeks (Fig. 8). By the 13-14th week, it is possible to identify the later generations of ureteral bud branches that will form each of the lobular collecting systems, which ultimately drain into the calyceal chambers in the papillae. The calyces are now evident as expanded broad chambers subtending a confluence of collecting ducts of nephron-bearing tubular branches (Fig. 9). Each of the primitive tubular branches emptying into the primitive calyces and their papillary ducts are sufficiently well delineated to resemble those of the mature kidney.

Formation of the Collecting System

The collecting systems of each of the renal lobes in the mature kidney are highly systemized. The medullary portion is comprised of nephron-free branches that collect the urine formed by aggregates of overriding cortical nephrons. The cortical nephrons formed within a renal lobe are organized in multiple aggregates or clusters. All their nephronic tubules connect with individual branches of the collecting ducts in the renal cortex (Fig. 1) and ultimately converge into a single papillary duct termination in a single renal papilla. The centrifugal arborization of each of these collecting tree units (i.e., starting with a single papillary duct) reflects the process of collecting duct branching that occurred during the early stages of nephrogenesis described above.

Dichotomous branching of the collecting system continues actively until about 20 weeks. The nephrons located in the inner cortical region in the mature kidney

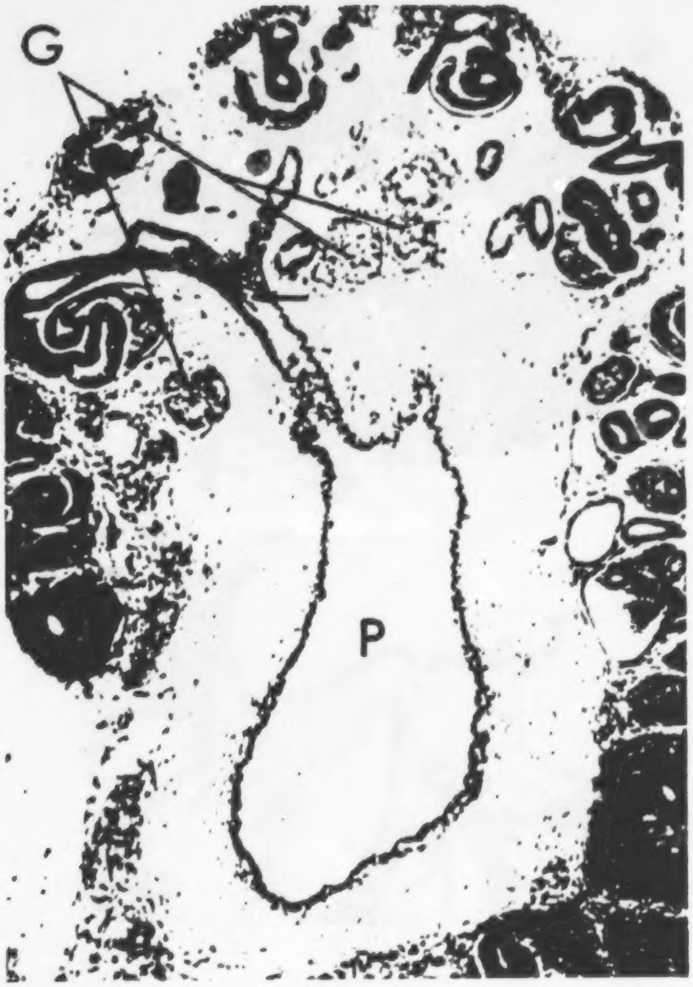

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Fig. 7. Kidney from 24 mm, 9 week embryo in cross-section. Early branches of ureteral bud are dilated to form primitive renal pelvis (P). Dilatation of proximal portions of next generation will leave terminal portions constricted (indicated by arrow) through which the calyces will communicate with pelvis. Of 18 well-formed glomeruli (G) present, 3 are visible in this section. [From: Potter EL (1972). "Normal and Abnormal Development of the Kidney." Chicago: Year Book Medical Publishers.]

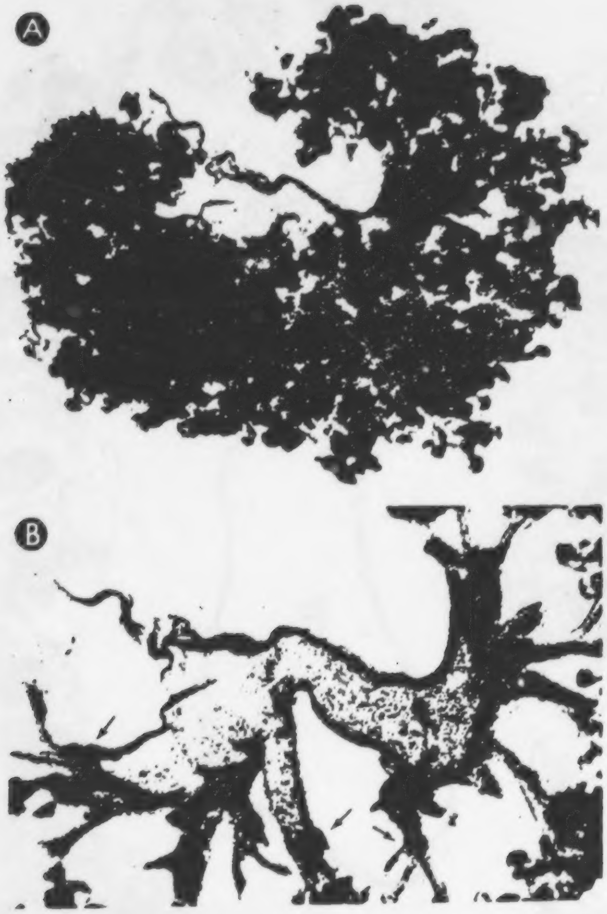

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Fig. 8. Kidney from 50 mm, 11 week human fetus. A. Entire kidney after preliminary microdissection. B. Most of the nephrons and collecting tubules have been removed to show ureter, renal pelvis, and earliest stages in formation of calyces. Tubules formed by third to fifth generations of ureteral bud branches have dilated to become primitive renal pelvis, and many large tubules empty directly into it. Initial evidence of calyceal formation is indicated by arrows. Note small diameter of ureter. [From: Potter EL (1972). "Normal and Abnormal Development of the Kidney." Chicago: Year Book Medical Publishers.]

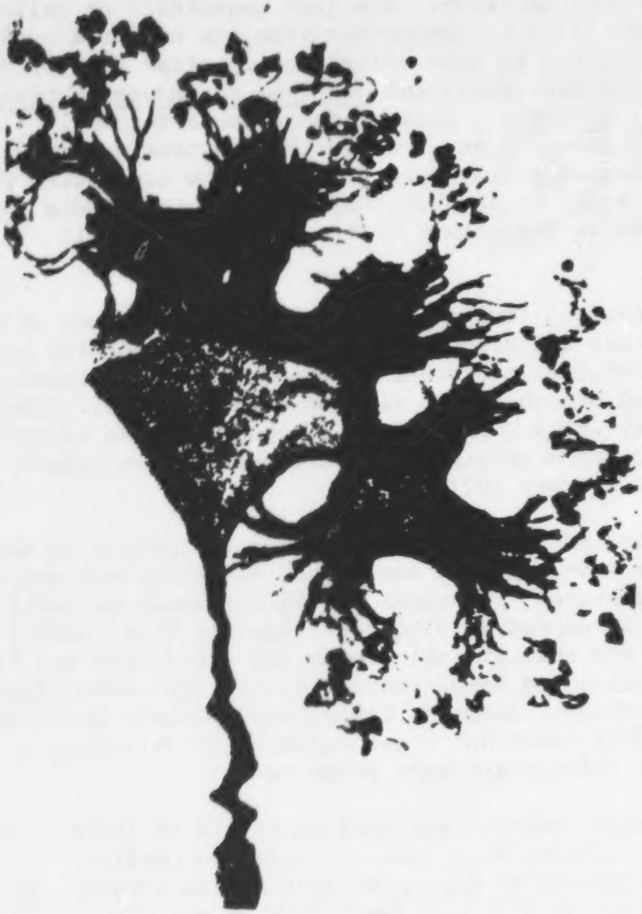

Fig. 9. Kidney from a 74 mm, 13 week human fetus. Part of the ureter (lower right), interpolar portion of the renal pelvis, and two minor calyces are shown, as well as the total length of a few collecting tubules and two nephrons. The calyces are more distinctively cup-shaped than in earlier stages and are beginning to assume a definitive form. Five to six generations of collecting tubules are present distal to those that dilate to form the renal pelvis. [From: Potter EL (1972). "Normal and Abnormal Development of the Kidney." Chicago: Year Book Medical Publishers.]

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(juxtamedullary) are those formed during the period of collecting duct division. The last generation of collecting ducts formed (14-15th generation from the ureteral bud), however, continue to grow centrifugally after collecting duct division has ceased and *continue to induce nephrons until about 32 weeks*. Extensions of these terminal nondividing generations of collecting systems grow toward the outer cortex in the form of spear-like tips which form arcades of nephrons that will be found in the middle and outer cortex in the mature kidney (about 80% of all nephrons).

A surprising finding of microdissection in the 20-week kidney is that all branches of the medullary portion of the collecting system, representing the 7-13th generations of ureteral bud branches, are nephron free (Fig. 10). The mechanism by which nephrons are cleared from the earlier branches to the more peripheral branches is not clearly understood (McCrory 1972).

The last period of renal development (period 4) begins at 32 weeks when ampullae disappear, signaling the end of nephron formation. The considerable increases in renal mass that occur in period 4 (3-fold increase in size) result from glomerular and interstitial growth and elongation and final differentiation and maturation of collecting ducts, distal nephronic tubules, loops of Henle, and proximal convoluted tubules. This exuberant organ hypertrophy is reduced or absent when infants are born prematurely.

Dysgenetic factors are very difficult to identify or define, and consideration must include the possible dysgenetic effects of increased hydrostatic pressure if obstruction to urinary outflow occurs at any time during development. The formation of the pelvocalyceal system and renal lobes and the beginning of nephron formation are synchronous with the separation of the ureter from the mesonephric duct and formation of the bladder and trigone. Opportunities for the association of malformations of the urinary tract with serious dysgenetic changes involving the parenchyma clearly exist from the 5-6th to the 14-15th week.

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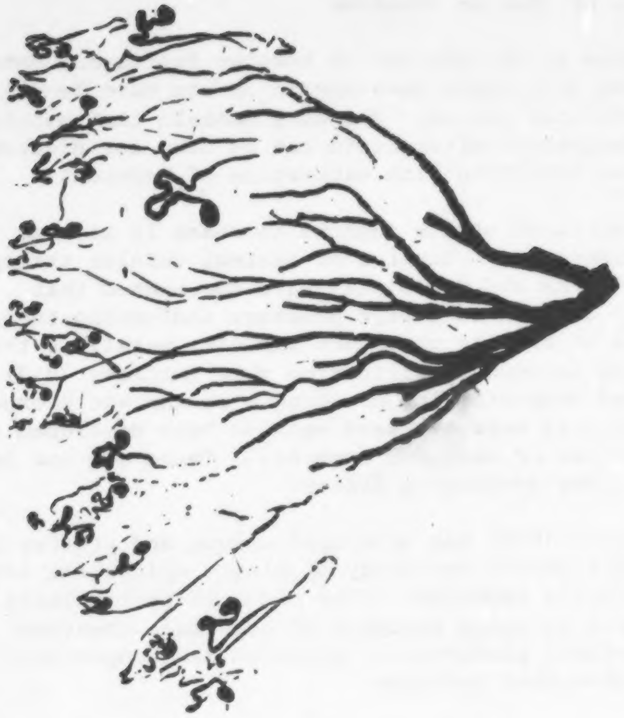

Fig. 10. Collecting tree at 5 months. The tubules have divided nine times in most instances, thus forming the fifteenth and final generation of the ductal system, i.e., its closed divided portion has thus been established. The divisions are now all bidfid. All the nephrons have been transported (Peter's transport process) to the periphery, and a separation into cortex and medulla is apparent. The nephrons appear in all configurations, from vesicles to those with well-developed loops of Henle; the former occur only at the periphery on the terminal budding ampullae. Note that every nephron is attached to an ampulla either directly by its connecting tubule or by an arcade; none has lagged behind. The kidney contains about one-third the ultimate number of nephrons. The cortical region as it exists at this time will form only the juxtamedullary zone of the definitive cortex. [From: Oliver J (1968). "Nephrons and Kidneys: A Quantitative Study of the Developmental and Evolutionary Mammalian Renal Architectonics." New York: Hoeber Medical Division, Harper and Row.]

Maturation of Tubular Function

Studies of the pattern of tubular functional maturation that occurs with renal development in man have been limited to the postnatal period. The many mammals that continue with nephrogenesis after birth can be used for studies of the changes occurring with maturation of nephrons.

All portions of the nephron increase in size as function increases. Studies of maximal tubular transport capacity (T_mPAH and $T_m$glucose) have documented that glomerular balance is always present, indicating that maturation of tubular transport capacity parallels the increase in glomerular filtration rate (McCrory 1972). We have a good understanding of changes at the whole-organ level, but only recently have methods been developed that permit studies of isolated segments. These are now being applied to the developing kidney.

Horster (1978) has developed unique and precise micro methods that permit the study of single epithelial segments from developing nephrons. This model is particularly suitable for _in vitro_ analysis of physical, chemical, and ultrastructural phenomena in perfused and nonperfused isolated dissected nephrons.

Horster (1978) reported studies of the differentiation of salt and water transport in the proximal convoluted tubule and in the loop of Henle in the neonatal dog, rat, and rabbit. He confirmed the existence of structural heterogeneity in the nephrons of the neonatal mammalian kidney at the cellular level. The most mature nephrons (typified by the juxtamedullary nephrons) have the functional and ultrastructural competence of mature nephrons, whereas the most immature nephrons (typified by the superficial subcortical nephrons) are the least advanced in differentiation. His data describe the cellular processes underlying the development of the prime functional attribute of this part of the nephron: active solute (sodium) transport and passive isosmotic movement of water permitting isotonic reabsorption of filtrate. Similar studies in the thick ascending loop of Henle, where sodium and chloride are characteristically reabsorbed without equivalent movement of water (thus diluting tubular fluid), document how this tubular segment changes by enhancing the transport rate of transtubular solute while maintaining low water conductance, thus becoming a diluted segment. The loop of Henle segments that transfer water passively (thin ascending and descending limbs) extend by longitudinal growth into the increasingly hypertonic medullary interstitium. The unique contribution of the development of the capacity for anisosmotic sodium and chloride transport in the thick ascending limb to the development of a functional countertransport system is obvious.

Horster and Zink (1982) extended their studies to describe the functional differentiation of the medullary collection tubules and the influence of functional maturation of the response to vasopressin. These studies demonstrated that, as the medullary collecting tubule differentiates, its responsiveness to ADH in terms of basal hydraulic conductivity coefficient changes. It is nonresponsive to ADH during early epithelial differentiation (Fig. 11), whereas the response of the mature epithelium is greater than that of other collecting tubule segments. These studies suggest that the differentiation of the unique capacity for water and ion transport in the medullary collecting tubule and its responsiveness to ADH also contribute to the increasing efficacy of the medullary countercurrent system.

The kidney not only plays a major role in maintaining fluid and electrolyte homeostasis in man but is also an important organ for the excretion of drugs and toxins. The processes underlying the maturation of these important functional abilities have received little investigative attention, even though impaired renal excretion of drugs and toxic chemicals clearly could have serious adverse effects on the neonate. The fetus is protected because the placenta serves as the fetal kidney.

Maturation of Organic Anion Transport

Hirsch and Hooke (1969) reported that renal p-aminohippurate (PAH) transport (a model system for organic acid transport) in rabbits increased rapidly from birth to 4 weeks of age and then declined to adult levels. Exposure of the young rabbit to penicillin by administration to the pregnant doe or to the newborn rabbit stimulated the developing transport system. The presence of substrate during renal development thus enhanced the rate of increase

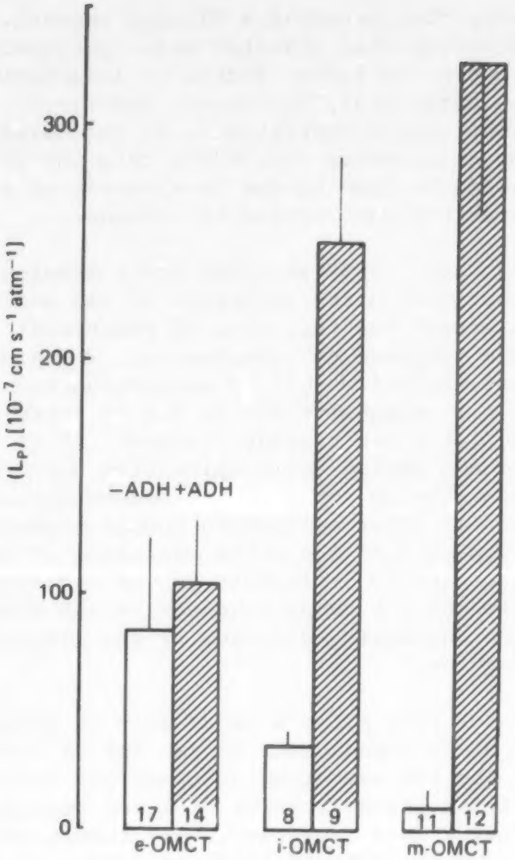

Fig. 11. Osmotic hydraulic conductance (L_p) of the outer medullary collecting tubule (OMCT) at three stages of ontogenic differentiation. Values represent mean ± S.E.M. of all data during the plateau phase of basal (-ADH) or activated (+ADH) hydraulic conductance. [Reprinted with permission from: Horster MF, Zink H (1982). Functional differentiation of the medullary collecting tubule: Influence of vasopressin. Kidney International 22:360-365.]

in transport activity (presumably by substrate-induced activation of the transport mechanism) and was associated with evidences of increased cellular protein synthesis. It was not possible to distinguish, by renal-slice uptake studies, the relative contributions to this increased transport activity of 1) increased intrinsic cellular or membrane carrier transport capacity, and 2) increased size of the tubules. Schwartz et al. (1978) recently reported studies providing an estimate of the contributions of these two processes. They used the isolated single tubule perfusion system to study PAH transport in the isolated perfused proximal straight tubules. They found that most (67%) of the increase in transport activity resulted from increased intrinsic activity, while about 33% was a consequence of the increase in surface area due to tubular growth. This suggests that substrate stimulation augments in some way the rate of maturation of the intrinsic cellular membrane transport systems rather than altering the age-related growth changes.

These interesting findings suggest that drugs ingested by the pregnant mother that pass via the placenta to the fetus could have pharmacological effects on fetal renal transport activities. This opens a broad area for research: studies of the effects of exposure of the pregnant animal to drugs, toxins, etc. on the pattern of normal renal functional maturation in late intrauterine life, and similar studies of postnatal ontogeny in species in which nephrogenesis continues after birth. The consequences of such exposure would obviously depend on the pharmacological and/or teratogenic properties of the agents involved. The developing mammalian kidney could provide a means of investigating this field, and a number of different models (isolated perfused tubules, kidney cell cultures, single nephron micropuncture) are available.

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CHEMICALLY INDUCED POLYCYSTIC KIDNEY DISEASE

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INTRODUCTION

Campbell (1951) noted in a large postmortem study in a general hospital setting that polycystic kidney disease (PKD) was present at autopsy in 1 in 260 adults and 1 in 220 children. PKD is the third most common cause of end-stage renal disease in patients presenting for dialysis. Not all cases of PKD are lethal; however, a large proportion of patients who present for dialysis and transplant have PKD. Over the last 15 years, PKD has represented an increasing proportion of end-stage kidney disease, perhaps because of the decreasing incidence of end-stage glomerulonephritis now seen in the western world rather than because of a true increase in incidence. For example, in a report from one large general hospital, PKD has become the second most common cause of terminal renal failure in patients on hemodialysis (Williams, Price 1970). The pathogenesis of PKD deserves closer attention.

Currently, there are several confusing classifications of PKD in use in medicine. Osathanondh and Potter's classification of the mid 1960's (1964) is still quoted widely, yet Potter in her recent book (1972) added several types of PKD outside her own numbering system. Many pathologists still place all their cases within the original five classes of Osathanondh and Potter. One of us (J.F.S.C.) occasionally sees cases that cannot be placed in any of the currently used classifications, including the one personally proposed in the early 1970's (Crocker *et al.* 1971). It is obvious that -- rather than carry on in a descriptive manner -- we should attempt to understand biochemical pathogenic mechanisms and center our classification on a stronger cornerstone than description. Unfortunately, we are not at this stage yet.

The genetic and physiological milieu for the induction of cysts is often difficult to study in the human situation because human breeding systems cannot be manipulated experimentally and because many of our cases are not diagnosed until the disease is at the end stage. Experimental models induced by teratogens may lead us to understand the mechanisms by which cyst formations can occur. In the following, we will discuss two forms of experimental PKD: 1) glucocorticoid-induced PKD, and 2) PKD related to diphenylamine.

GLUCOCORTICOID-INDUCED POLYCYSTIC KIDNEY DISEASE

Thelma Baxter (1960) first noted the development of cyst formation in the kidneys of young rabbits after exposure to glucocorticoids. She microdissected these kidneys and found that their structure has features that are seen in the human condition in both the adult form of PKD and in the cystic dysplasia lesion. Glucocorticoid-induced cysts are in the collecting tubules and in the subcapsular zone of the renal cortex in the rabbit, but in the mouse the cysts may be more diffuse and extend deep into the cortex. A second form of cyst consisting of a dilatation of Bowman's capsular space has been recorded, and Ojeda and

Garcia-Porrero (1982) recently noted that these capsular cysts have a parietal layer that may have been composed of podocytes. They theorized that "an inhibitory factor that stabilizes the phenotype of the parietal cells under normal conditions is absent under conditions that cause glomerular cyst formation."

This form of PKD is induced by injecting newborn rats, rabbits, hamsters, or mice with a certain form of steroid. The fact that these animals all have nephron differentiation continuing postnatally may be why we are able to induce cysts, as induction of this disease has never been reported at a stage in an animal species where nephron differentiation is complete. Vlachos (1972) studied the long-term effects of this disease in young rabbits, noting that some of the lesions were reversible if steroids were discontinued. However, in our experience, many of the animals have such severe PKD that death with uremia intervenes.

Although studies have illustrated that the developing nephron may be structurally altered by glucocorticoids, there appears to be a complex interrelationship of steroids during pregnancy. The fact that cortisone is a prominent steroid in the human placenta, in the fetal plasma, and in the amniotic fluid (Murphy 1978) is paradoxical because it is also known that high cortisol levels in early fetal life in many species can cause malformations and have a detrimental effect on early development. In late pregnancy, when the cortex of the kidney is differentiating, cortisone and cortisol levels decrease, which allows normal maturation to occur. Interestingly, Leiberman (1976) has suggested that, in human infantile PKD, cyst formation only occurs *in utero* and does not continue to be induced postnatally.

The development of *in vitro* techniques for the isolation and perfusion of individual renal tubules has contributed significantly to our knowledge of the functional and structural characterization of many segments of the nephron. Because environmental conditions can be controlled in these experiments, it is now possible to study the action of acute hormonal exposure. *In vitro* studies have shown that the electrolyte composition of the diet on chronic exposure to corticosteroid hormones can significantly alter the transepithelial potential difference and rates of Na^+ and K^+ transport (Wade *et al*. 1979). Structurally, the mammalian cortical collecting tubules consist of two cell types: principal or "light" cells, which are the most numerous, and a second, less numerous class called intercollated or "dark" cells. In a study in rabbits treated chronically with corticosteroids, the steroids resulted in significant and specific amplification of the basolateral area of the principal cells (Wade et al. 1979).

In the rat kidney, there are at least three types of corticosteroid receptors delineated by a variety of physiological and biochemical studies (Feldman, Funder 1973). Type I is designated as the mineralocorticoid site, and types II and III are designated as the glucocorticoid sites. These glucocorticoid receptor sites may be identical with certain transport sites and thus be involved in altering the interluminal transport. In 1969, both Tomkins et al. and Griffin and Ber reported that the induction of enzyme activity by steroid hormones was a cell-cycle-dependent process. In a later study, Cidlowski and Cidlowski (1982) noted that, although cytoplasmic glucocorticoid receptors are present throughout the entire cell cycle, these are "dynamically" regulated so that they may allow active homone effect only at certain phases of the cell cycle.

The effects of glucocorticoids on the growth of renal tubular cells are little understood, but there are numerous reports that they affect the growth of animal cells in culture, either by stimulating or inhibiting cell division. Studies have shown that the glucocorticoid action only takes place if polypeptide growth factors or serum are added to the culture media. Thus, glucocorticoids may cause many of their actions by imitating the response of other homones (Baker et al. 1978).

Some years ago, we began studies on the pathological similarities of experimental hydrocortisone acetate (HCA)-induced PKD and human PKD to establish whether HCA-induced PKD can reasonably be regarded as a model system for the study of human PKD. Pregnant Sprague-Dawley rats were obtained from three different animal suppliers and injected with 250 mg/kg of hydrocortisone acetate. The results (Fig. 1) show considerable variation in susceptibility of the animals to HCA-induced PKD, depending on the source of supply of the rats.

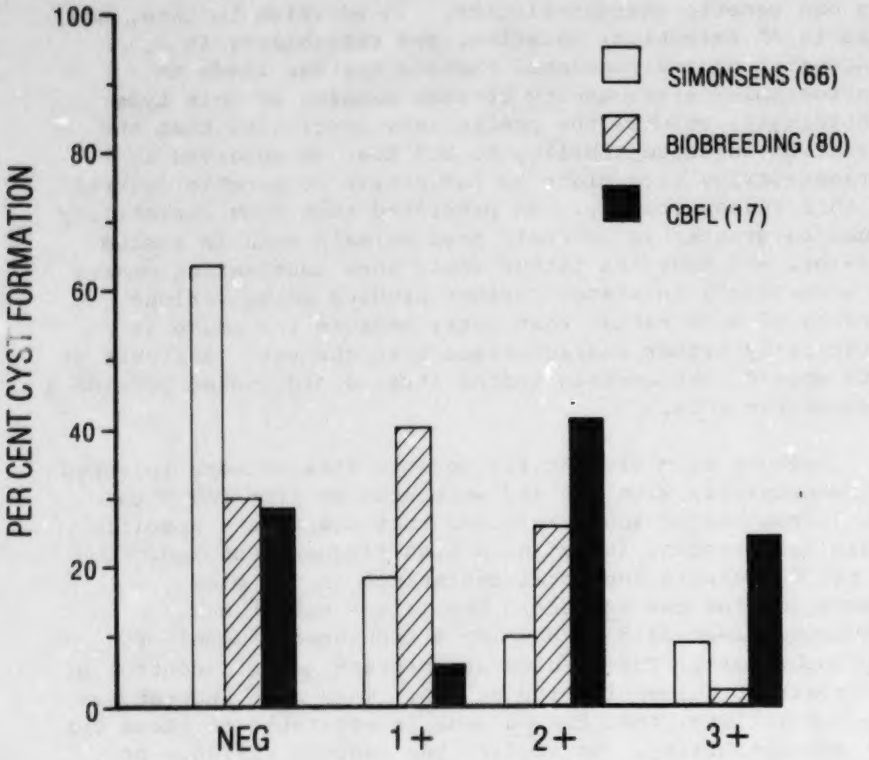

Fig. 1. Levels of polycystic kidney disease induced experimentally by injection of 250 mg/kg hydrocortisone acetate in Sprague-Dawley rats obtained from different suppliers.

Although these rats are said to be of the same "strain," they are likely to be genetically heterogeneous because they are propagated by random breeding, which maintains genetic variance at a maximum when the breeding number (N) is large. When different lines of the same strain are randomly bred in isolation at various centres, the genetic diversity between centres will tend to increase. Since random fixation of alleles at each locus is approximately equal to 1/2 N, random breeding in populations of finite and small size (such as those at each individual centre) will lead to each of these populations developing its own genetic characteristics. In addition to this, the effects of selection, mutation, and variability in nongenetic or environmental factors further leads to considerable heterogeneity between samples of this type. Accordingly, we drew the preliminary conclusion that the variation in susceptibility to HCA that we observed in Sprague-Dawley rats might be indicative of genetic control of this susceptibility. We predicted that such variability would be greater in randomly bred animals than in inbred strains, and that the latter would show contrasting means. We accordingly initiated further studies using various strains of mice rather than rats, because the mouse is genetically better characterized than the rat. Analysis of data showed that certain inbred strains did indeed provide a predictable model.

Newborn mice of specific genetic strains were injected intramuscularly with HCA and were studied from Day 5 to Day 12 post-injection. We found that there were specific stain differences, including a high frequency of induction in the C3H strain and total resistance in CBA mice homozygous for the kd gene. The latter mutant has previously been said to develop a spontaneous model of nephrophthisis. The results demonstrate genetic control of the trait of susceptibility to HCA. They also suggest, as one possibility, that the kd gene is epistatic to genes for HCA susceptibility. We studied the genetic variance of sensitive and insensitive strains and the F_1 and F_1 offspring of their crosses. From those experiments we derived estimates of the degree of genetic determination of the trait (Crocker, Blecher 1982). Our preliminary results support the contention that there is a significant genetic susceptibility in this model. It is being increasingly appreciated that an understanding of genetic factors in the development of PKD is an essential prerequisite to rational classification of this group of diseases as a basis for further studies and ultimate therapeutic advance. A broad overview of genetically different forms of PKD in humans has been given by Schmike (1976). However, numerous problems -- such as the absence of consensus on the definition of the term "renal cyst" -- evidently still render unanimity in classification impossible.

On the basis of our experiments with the steroid model, we propose a "two-strike hypothesis." First, the mother would have to have prolonged production of the corticosteroid late in kidney differentiation; second, the fetal kidney would have to be susceptible. This hypothesis would accommodate many of the data available on infantile PKD, including the genetic phenomenon of variable penetrance within a family. With further work in our laboratories, we hope to demonstrate the feasibility of using this animal model to aid in understanding human PKD.

DIPHENYLAMINE

Diphenylamine (DPA) is widely used in industry as an antioxidant, dye mordant, and plastic stabilizer. Thomas et al. (1957) first called attention to the PKD on long-term feeding of DPA to adult rats. Kime et al. (1962) confirmed the renal teratogenic effects of DPA and noted the similarity of the lesions to PKD in man.

DPA-induced PKD is remarkable since the lesions develop in the mature nephrons of adult animals fed the DPA compound in a 1.5 to 2% dietary concentration for 6 mo to 1 yr. The same chemical fed to pregnant animals will then induce a disease in the offspring resembling infantile PKD, with cysts in the proximal nephrons or anywhere along the collecting system (Safouh et al. 1970).

Safouh et al. (1970) microdissected DPA kidneys and noted that the structural and functional changes were similar to adult PKD. Darmady et al. (1970) emphasized the similarities of this model to human disease. He proposed that desquamating cells from degenerating tubular epithelium led to cystic dilatation of contiguous tubular segments. However, Evan and Gardner (1976) recently performed scanning electron microscopy on DPA-induced PKD in adult rats and noted a hyperplastic response in one area of the collecting duct that caused structural changes leading to cyst formation by increasing the hydrostatic pressure in the tubular lumen. Cho et al. (1979) have since confirmed Evan's work and noted, in human adult-type polycystic kidneys, cellular hyperplasia like that seen in the DPA model. Milutinovic and Agodoa (1983) recently noted that the collecting duct cellular hyperplasia was found only in end-stage disease and not in biopsies from early cases. They speculated that there was a defect in "compliance" of the tubular basement membrane as previously suggested by other authors.

Several other organic chemicals -- such as 5,6,7,8-tetrahydrocarbazole-3-acetic acid, biphenyl, polychlorinated biphenyls, (2,4,5-trichlorophenoxy)acetic acid, and 2-amino-4,5-diphenylthiazole -- have been reported to cause similar polycystic lesions in animals. Therefore, it would appear that the collecting duct is susceptible to cellular hyperplasia when chronically exposed to certain chemicals. Hong et al. (1974) showed DPA to specifically inhibit both the active transport of sodium across toad skin and the antidiuretic-hormone-induced passive transport of water across the toad's urinary bladder. They theorize that this may link with the collecting duct cyst formation and the lack of concentrating ability seen in DPA-fed rats.

Thus, the DPA model, a model similar to the adult human PKD, appears to also require two parts: 1) cellular hyperplasia at the base of the cyst and perhaps an altered tubular basement membrane, and 2) change in hydrostatic pressure in the lumen of the tubules by alternating tubular ion transport.

DPA can induce functional lesions primarily in the collecting duct of an adult rat (such as a defect in maximal urinary concentrating ability) at a time when gross pathological defects may not be discernible (Eknoyan et al. 1976). This may be similar to the finding that family members of patients with polycystic kidneys cannot maximally concentrate their urine, suggesting that there is a primary defect in the transport of ions in the collecting ducts.

In earlier studies, we found that certain brands of DPA could induce PKD in offspring if fed to the pregnant mother rat. It was later found that the active chemical needed to induce PKD in the offspring was not DPA but an oxidative product $N_1N_1N_1$'-triphenyl-p-phenylenediamine, which is related structurally to the carcinogenic amines (Safe et al. 1977).

Hemodialysis and peritoneal dialysis are important therapeutic advances of the last half century. Unfortunately, each new progress has caused the appearance of new problems. Dunnill et al. (1977) first described the occurrence of 14 cases of acquired PKD on autopsy of 30 cases in which dialysis had been the course of treatment (mean dialysis time of 3 to 4 yr). Clinical, pathological, and radiological investigations of these patients when they first presented did not reveal any evidence of renal cystic change. Six of the cases had also developed renal tumors, five of which were multiple neoplasms. Although the association was rarely noted before, since Dunnill's report there have been several other reports linking long-term hemodialysis with PKD and tumors (Table 1). McManus and Hughson (1979) have noted restructuring of the kidney during hemodialysis. Hughson *et al*. (1980) note that "accumulating data suggest that the growing population of patients on life saving maintenance hemodialysis may be at risk of developing renal cancer. Carcinomas arising in this group of patients have been noted to occur on a background of acquired cystic disease." The fact that such effects have not been noted in previous teratogenic animal experiments may be because of the short term nature of such studies.

A *Lancet* editorial (1977) stated that "it is surprising that such a dramatic and apparently frequent condition has not been reported before, since, during the past decade, the kidneys of many thousands of dialysis patients must have been examined." The same editorial also noted the "intriguing possibility" that this form of cystic disease is the same disease that has been reported previously in animals on exposure to teratogenic chemicals.

Flexible polyvinyl chloride (PVC) has replaced the older rigid material in many applications in medicine. High-molecular-weight polymers like PVC are made flexible by interfusing plasticizers, most commonly phthalic acid esters (e.g., di[2-ethylhexyl] phthalate, known as DEHP). A variety of other substances, known as heat stabilizers, antioxidants, inhibitors, catalysts, and filters, are also added during the manufacturing process of these flexible plastics.

Ono *et al*. (1975) first noted that DEHP was leaching from the hemodialysis tubing. Their report was followed by that of Gibson *et al*. (1976), who noted in their patients a steady increase with the length of hemodialysis. Chen *et al*. (1979a) studied the clearance of DEHP from nonuremic patients undergoing treatments for psoriasis and also from dogs who were in various stages of renal failure. DEHP was detected in some of the 24-h urine specimens from nonuremic patients undergoing therapy. The DEHP concentrations ranged from nondetectable to 159.8 μg/24 h. They found "no correlation between serum levels of DEHP and either type of

Table 1. Clinical Reports on Association Between Dialysis and Polycystic Kidney Disease

Reference	Cystic Kidneys/ Total Number Examined	% Cystic Change	Mean Dialysis Time (mo)	Tumors
Bansal *et al.* 1980	5/52	10	74	1
Bommer *et al.* 1980	11/14	79	62	?
Dunnill *et al.* 1977	14/30	47	40	6
Fayemi *et al.* 1980	24/80	30	51	9
Hughson *et al.* 1980	20/66	30	35	6
Ishikawa *et al.* 1980	10/23 23/29	43.5 79	<3 yr >3 yr	4
Krempien, Ritz 1980	21/22	95	33	4
Mirahmadi, Vaziri 1980	15/32	44	43	0
Ohmori *et al.* 1981	1/1		10 yr	1

artificial kidney used or therapeutic modality." Uremic dogs, however, were unable to clear the DEHP. Perhaps normal renal function is necessary for the excretion of nonmetabolized DEHP.

In another study, Chen et al. (1979b) studied tissue levels of DEHP in uremic patients who had been on long-term hemodialysis and found the chemical to be present in the tissues of some patients.

Lewis et al. (1978) studied the time sequence of accumulation of DEHP in patients on hemodialysis and concluded that "perfusion of whole blood through arterial-venous tubing (that supplied with a dialyzer) for one h in vitro yielded 3.23 mg of the compound, while similar perfusion of the tubing plus the artificial kidney yielded 6.10 mg. Its mean concentration after dialysis in patients undergoing hemodialysis was 751 µg/L of serum." A significantly higher level of DEHP was present in patients who had received 50 or more hemodialysis treatments than in patients who had received less than 50. One group of patients showed a maximum peak in 3 h; another group showed a steady increase until the end of dialysis. The DEHP was rapidly cleared from the blood, with the largest decrease at 5 to 7 h after dialysis.

It would be interesting to know whether either the group that metabolizes the chemical (perhaps to a more toxic compound) or the group that does not metabolize these plastic products relates to the clinical situation of hemodialysis-induced PKD.

Although DEHP is not known to cause PKD, it can be used as a marker for other chemicals. Lewis et al. (1978) noted "Plastic tubing contains other additives besides plasticizers, such as anti-oxidants, ultraviolet stabilizers, and fire retardants. These compounds do not have as great a tendency to leach out as does DEHP; however, they are soluble in DEHP and so may accompany it."

Another source of these chemicals for patients on hemodialysis and peritoneal dialysis is the soft plastic bags used for blood, dialysate, and intravenous products. We know that organic chemicals are leached from the plastic and into the parenteral products.

The acknowledged body burden of plasticizers in man is associated with a widely dispersed environmental pool accumulated from nonmedical products. Hemodialysis patients, on the contrary, would appear to accumulate the majority of their plasticizers from their contact with medical products. Several years ago, the question of the safety of phthalate ester plasticizers came to the fore following a report by Jaeger and Ruben (1972) of phthalate plasticizers found in blood stored in plastic blood bags and in tissues of patients who had received blood transfusions. So far, no toxic effects from the accumulated residue in the liver, lung, and spleen of the humans have been reported. Problems such as peripheral neuropathy and other idiopathic symptoms have been stated to be associated with DEHP, but strong experimental substantiation does not exist. However, there is evidence of toxicity in some studies -- particularly long-term studies in animal and tissue cultures.

We hypothesized that the post-hemodialysis-acquired PKD could possibly be the human equivalent of the DPA animal model. The unique feature would be that the animal model was known before the human disease. We obtained from a nephrectomy a kidney from a patient on long-term hemodialysis. This patient had developed polycystic kidneys following dialysis therapy and did not have PKD prior to the start of hemodialysis, so it is presumed that this patient acquired PKD. The kidneys were subjected to an extraction process and the residue was analyzed by gas chromatography using a flame ionization detector. On the original analysis, a peak similar to DPA was demonstrated. Confirmation was then attempted using another column, and the peak was found to be not DPA. Further work on identification is under way.

To further test our hypothesis that plasticizers leach from hemodialysis tubing, we are testing plastic equipment in rats. A leachate of the dialysis tubing was obtained, and 120 young Sprague-Dawley rats divided into five groups are being tube-fed three times a week. Rats in each group receive either one of two doses of leachate, DEHP, the vehicle, or water. Dosages were calculated to approximate human dialysis exposure on a weight basis. Every 3 mo, urine and blood samples are taken from selected rats from each group and analyzed for renal function, and animals are killed randomly for morphological examination. Microscopic examination is done in a double-blind fashion. Preliminary results are that all kidneys from the groups exposed to high-dose leachate or DEHP showed tubular ectasia in the first 6 mo. However, there were also two positive controls in the vehicle group that showed some degree of tubular ectasia. These animals are being examined for possible contamination by chemical analysis of tissue. Water controls were entirely negative. The degree of ectasia in the experimental group varied directly with the dose of chemical injected. In summary, this preliminary work suggests that long-term exposure to plasticizers from hemodialysis equipment may play a part in the PKD seen in the human.

It is increasingly obvious to us that experimental animals may give an explanation as to how cysts form, and lead to a more logical classification of the human disease.

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THE CONTRIBUTION OF THE KIDNEY TO THE RETENTION OF SODIUM DURING GROWTH

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INTRODUCTION

The process of growth is intimately related to the retention of a variety of substances, including sodium. Balance studies have demonstrated that about 70% of the sodium provided in the diet is retained by the breast-fed puppy (McCance, Widdowson 1958) and about 30% by the healthy premature infant fed regular formula (Gordon *et al*. 1939). The tendency of the newborn to retain sodium, which is appropriate under physiological circumstances, persists, however, when the sodium load is increased. In one study (Janovsky *et al*. 1967), 6 infants of between 14 and 42 d of age were given initially a diet containing about 5 meq sodium/d and then switched to a diet containing 100 meq/d. This resulted in a 10-fold rise in the clearance of sodium, from 0.08 and 0.8 ml/min/1.73 m^2. However, the fraction of sodium that was reabsorbed remained the same, at about 60%, resulting in a marked increase in the retention of this ion. As a consequence, all the infants gained an inappropriate amount of weight and the majority became edematous. A similar response was observed in 5- to 7-mo-old infants who were fed for 2 wk diets containing either 50 or 80 meq sodium/kg food (Whitten 1969).

Retention of inappropriate amounts of sodium is observed in infants not only following chronic administration of large amounts of salt but also when a load of salt is administered acutely. This has been demonstrated by Dean and McCance (1949) in humans and by Goldsmith *et al*.

(1979) in dogs. In the latter study, the response of puppies to a load of sodium equal to 10% of body weight was compared to that observed in adult animals. During the 2 h following administration of the saline solution, the puppies excreted about 5% of the total load while the adult dogs disposed of about 50% (Fig. 1).

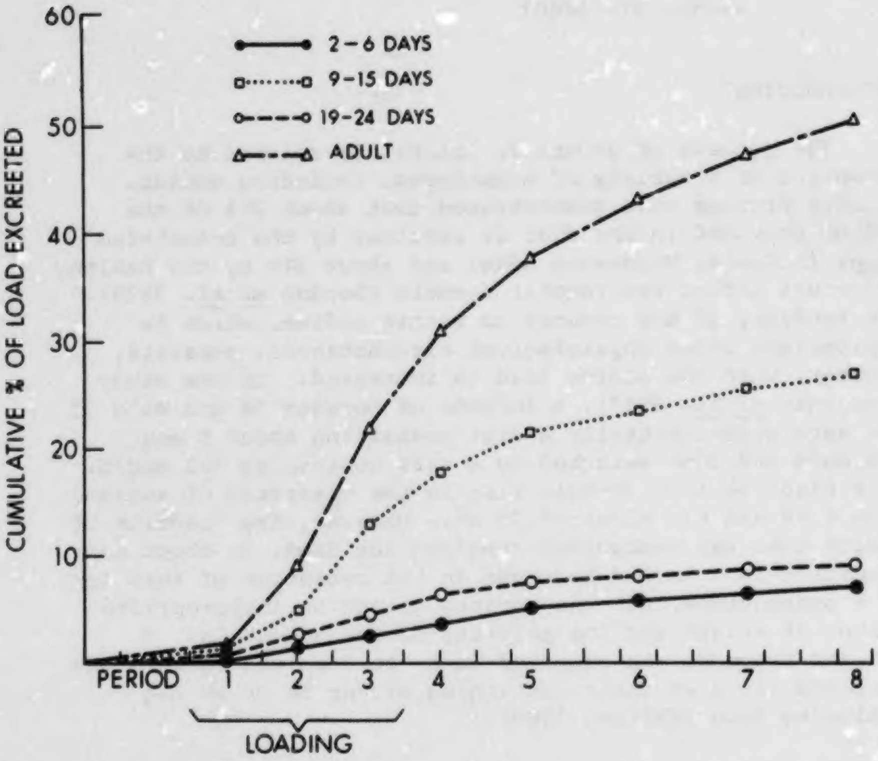

Fig. 1. Cumulative sodium excretion over a 2-h period of observation in dogs of various ages subjected to an acute load of normal saline solution equal to 10% of body weight.

GLOMERULAR FILTRATION RATE

It has been postulated that the main factor responsible for the retention of sodium during early postnatal life is the low rate of glomerular filtration (GFR). It is, however, rather obvious that this variable cannot account entirely for the retention of sodium observed under conditions of sodium loading, since a decrease in the fractional reabsorption of sodium of only 1% will be sufficient to restore the balance of sodium within a few hours. The initial increase in GFR consequent to the acute expansion of the intravascular volume was of relative similar magnitude in the newborn and the adult animal (Goldsmith et al. 1979). However, while in the adult dogs GFR remained at high levels throughout the entire period of observation, in the puppies GFR returned to the baseline values by the end of the loading period. Yet, when the sodium excretion was corrected for differences in GFR (Fig. 2), the age-related differences persisted. This indicates that factors other than GFR (i.e., tubular reabsorption) contribute to the retention of sodium seen in the neonate.

PROXIMAL VERSUS DISTAL REABSORPTION

The segment of the renal tubule where the majority of the sodium is reabsorbed is the proximal tubule. It was therefore appropriate to investigate whether or not this segment is involved in the enhanced reabsorption of sodium observed in the growing animal and human. Micropuncture studies done in puppies (Horster, Valtin 1971) and in guinea pigs (Spitzer, Brandis 1974) ruled out this possibility. In neither of these species did the tubular fluid to plasma inulin ratio (TF/P_{IN}) change by the end of the proximal tubule as a function of age, indicating that similar fractions of the filtered sodium and water are reabsorbed in this segment of the tubule both in the newborn and in the adult subject (Fig. 3). This demonstrated that the increase in GFR that occurs during development proceeds in parallel with the increase in the transport capacity of this tubular segment, resulting in the maintenance of the glomerular tubular balance. Under conditions of volume expansion, the fractional reabsorption in the proximal tubule was lower in the newborn than in the older animal, indicating again that

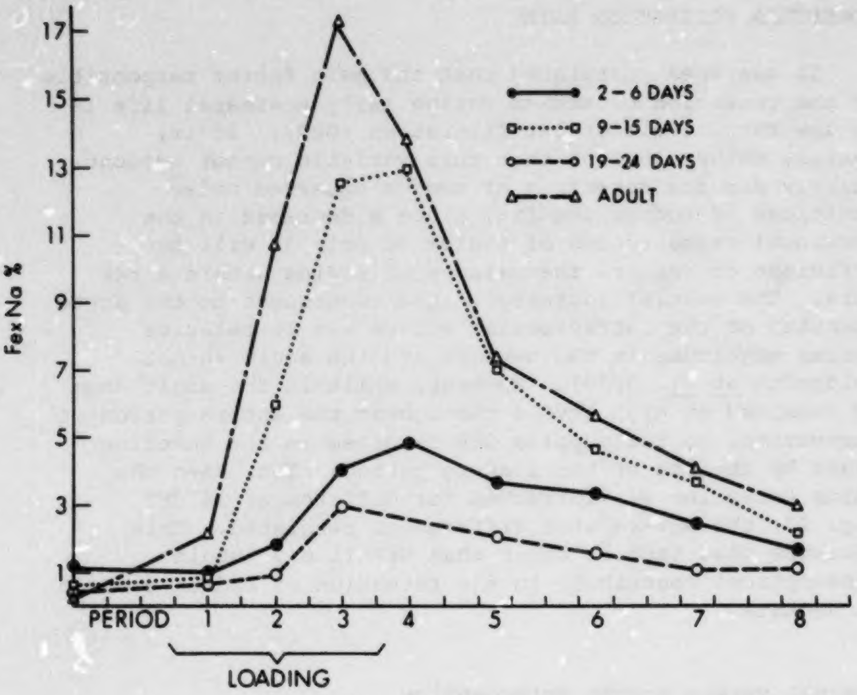

Fig. 2. Changes in the fractional excretion of Na^+ (FexNa) in dogs of various ages subjected to acute administration of a solution of normal saline equal to 10% of body weight.

this is not the place where the retention of sodium occurs at early age.

That tubular segments located beyond the proximal tubule may indeed be involved in this phenomenon was suggested by clearance experiments in 1- to 23-d-old puppies (Kleinman 1975). This investigator administered drugs known to block the reabsorption of sodium in the distal segments of the nephron (chloratiazide and ethacrynic acid) prior to and following the administration of a load of saline solution corresponding to about 3% of body weight. Since, during saline expansion, newborn dogs excreted less of the filtered sodium than the adult animals, and since the change in proximal fractional reabsorption appeared to be similar

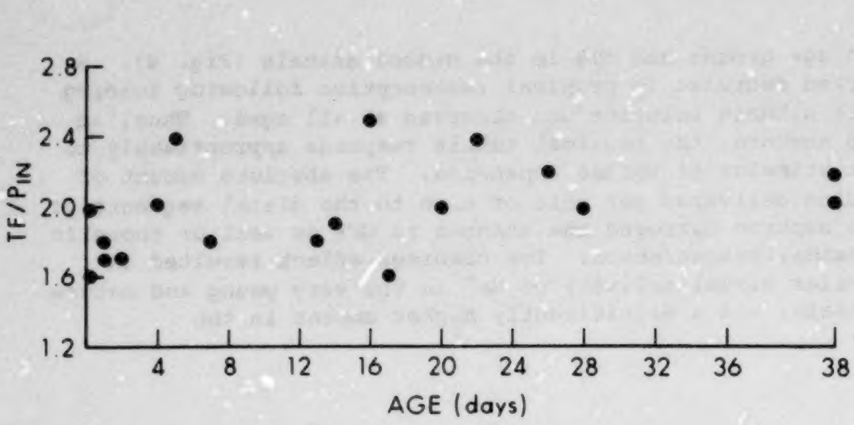

Fig. 3. Fractional reabsorption of fluid (TF/P_{IN}) by the proximal convoluted tubule of guinea pigs of between 1 and 38 d in age.

in animals of all ages, the author surmised that the distal tubules of the newborn dogs must have absorbed a greater fraction of the filtered load than the distal tubules of the adults.

A more precise exposition of the behavior of various segments of the nephron during development was provided by some micropuncture experiments we performed in guinea pigs (Schoeneman, Spitzer 1980). Samples of end proximal tubule fluid were obtained by the recollection technique under hydropenic conditions and following expansion of the intravascular volume with an isotonic albumin solution corresponding to 5% of body weight. The amount of sodium reabsorbed in the proximal tubule was estimated from changes in TF/P_{IN}, and the amount reabsorbed by the distal nephron was calculated as the difference between Na^+ present at the end of the proximal tubule and that excreted in the final urine. Animals were divided in three age groups (1 to 14 d, corresponding to a period of development characterized by low levels of superficial nephron GFR; 15 to 22 d, corresponding to a period of rapid rise in superficial nephron GFR; and >23 d, characterized by a slow rise in GFR).

Under control conditions, fractional reabsorption in the proximal convoluted tubule was about 40% in the first two age groups and 60% in the oldest animals (Fig. 4). A marked decrease in proximal reabsorption following loading with albumin solution was observed at all ages. Thus, in the newborn, the proximal tubule responds appropriately to the stimulus of volume expansion. The absolute amount of sodium delivered per unit of time to the distal segments of the nephron mirrored the changes in GFR as well as those in proximal reabsorption. The combined effect resulted in similar distal delivery of Na^+ in the very young and mature animals, and a significantly higher amount in the

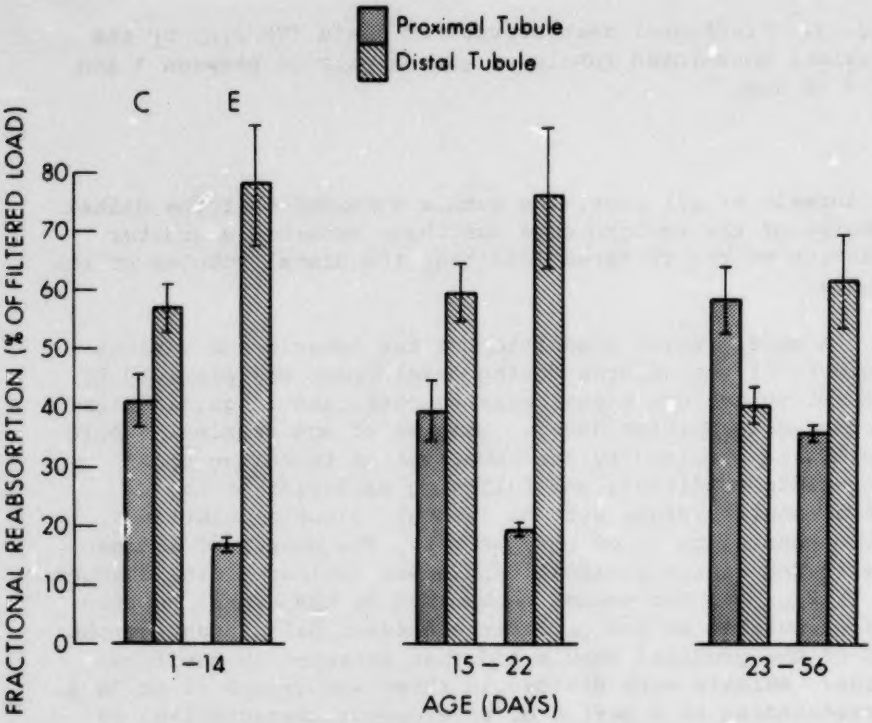

Fig. 4. Fractional reabsorption of Na^+ by the proximal convoluted tubule and by the distal nephron in guinea pigs of various ages. C = control, E = experimental (acute loading with an isotonic albumin solution equal to 5% of body weight).

intermediate age group. Distal reabsorption, expressed as a percent of the amount delivered into this segment, did not vary but was slightly lower during saline loading. As a consequence, the distal nephron had to reabsorb significantly more sodium in the 15- to 22-d-old guinea pigs than in the 1- to 14- or 23- to 56-d-old animals. It should be obvious that, under both control and experimental circumstances, distal reabsorption exceeded proximal reabsorption in the first two age groups, whereas the converse was true for the older animals in the hydropenic state. During volume expansion, close to 80% of the filtered load was reabsorbed by the distal tubule of the young; only 60% was reabsorbed by that of the adult guinea pigs subjected to comparable degrees of albumin loading.

RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM

There is ample evidence that adrenal cortical steroids exert a specific regulatory effect on the distal tubular transport of both Na^+ and K^+. Mineralocorticoids are thought to enhance the reabsorptive transport mechanism either by providing more energy to the active transport pump (Goodman et al. 1969) or by affecting the passive permeability of the tubular epithelium to sodium (Wade et al. 1979). There is ample evidence that the renin-angiotensin-aldosterone system is highly stimulated in the infant (Drukker et al. 1980; Kotchen et al. 1972), but no direct relationship has been established between the high levels of aldosterone prevailing during this period of life and the enhanced distal tubular reabsorption of Na^+. A causal link between aldosterone levels and the positive sodium balance of the growing infant was suggested by our experimental observation of a close relationship between the drop in plasma renin activity and the amount of sodium excreted in the urine (Drukker et al. 1980; Goldsmith et al. 1979). It should be noted that the 2-wk-old puppies excreted more sodium than the 3-wk-old animals (Fig. 2), showed the highest levels of renin prior to loading, and underwent the largest absolute drop in plasma renin activity following the infusion of saline. In further studies performed in puppies and adult dogs (Fig. 5), we were able to demonstrate a direct quantitative relationship between the fall in plasma aldosterone consequent to the expansion of the intravascular space and the amount of natriuresis occurring under these circumstances (Ito et al. 1983).

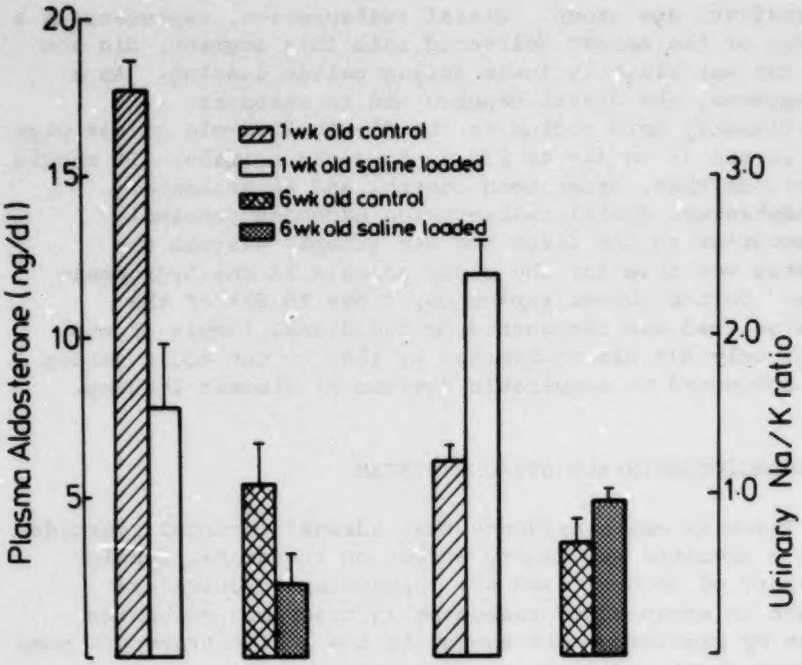

Fig. 5. The effect of sodium loading on plasma aldosterone concentrations and urinary Na/K ratio in newborn and young dogs. Note the inverse proportional relationship between the changes in these two variables.

Furthermore, an inverse relationship obtained between the levels of aldosterone in the plasma and the sodium/potassium ratio in the urine, indicating that the tubule of the newborn is responsive to aldosterone.

The status of the renin-angiotensin-aldosterone system can also account for the inappropriate sodium loss observed to occur in premature infants. Sulyok et al. (1979a, 1979b) performed balance studies and simultaneous measurements of plasma renin activity, plasma aldosterone concentration, and urine excretion of aldosterone in 1-wk-old neonates of gestational ages between 30 and 41 wk. The infants born at between 30 and 32 wk of gestation were found to have the highest urinary sodium excretion and to be in a negative sodium balance despite very high levels of plasma renin activity and high levels of plasma aldosterone. In the group of infants born beyond 32 wk of gestation, a positive sodium balance was achieved while the plasma renin activity diminished slightly and the plasma aldosterone concentration remained at similar levels. These observations indicate that, in response to sodium wasting, premature infants can augment their plasma renin activity but their adrenals fail to respond appropriately to this stimulation. In addition, the renal tubules of these immature infants appear to be partially unresponsive to aldosterone. An increase of about 30% in Na^+ reabsorption by the distal tubule was observed in prematurely born babies between the first and second week of extrauterine life.

The fact that, in the newborn and infant, aldosterone can maintain an effect on renal reabsorption of Na^+ for an extended period of time is probably due to the incorporation of sodium in the growing cells. In the adult subject, the increase in the secretion of aldosterone leads to the expansion of the extracellular fluid volume which, in turn, depresses the reabsorption of sodium and water in the proximal segment of the nephron. A new balance state is reached that persists as long as the secretion of the hormone is high (August et al. 1958). In the growing individual, the expansion of the extracellular fluid compartment, which apparently is responsible for the establishment of the new balance, cannot occur because most of the sodium retained is deposited in the intracellular compartment (Forbes 1962). It is therefore appropriate to surmise that the deposition of Na^+ in cells creates a state of continuous stimulation of the renin-angiotensin-aldosterone system and that this, in turn, enhances the reabsorption of Na^+ at the level of the distal nephron, allowing the positive sodium balance. On the other hand, when the rise in aldosterone is insufficient, or when the distal tubule is unable to respond appropriately to hormonal stimulation, renal loss of sodium and negative sodium balance ensue.

The stimulation of the renin-angiotensin-aldosterone system prevailing in infancy may be related to the emergence of essential hypertension later in life. Since both the developmental increase in blood pressure and the onset of hypertension appear to be associated with changes in the vascular sensitivity to angiotensin (Muirhead et al. 1978;

Munoz-Ramirez et al. 1978), it is reasonable to postulate that excessive stimulation of the developing vasculature may result in an inordinate increase in sensitivity to catecholamines or angiotensin and may result in hypertension. An additional or alternate factor might be represented by an alternation in sodium balance. A continuous tendency towards a positive Na^+ balance may result in an increase in the affinity or in the binding capacity of the renal tubule for aldosterone. That this may indeed be the case is suggested by observations, in animals with genetically determined hypertension, of an excessive positive sodium balance during growth (Bianchi et al. 1975) or of high levels of blood pressure when a high-salt diet is provided during maturation (Dahl et al. 1968).

ACKNOWLEDGMENTS

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THE EFFECT OF PRENATAL PROTEIN-CALORIE MALNUTRITION ON KIDNEY DEVELOPMENT IN THE RAT

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INTRODUCTION

Both protein deficit (Zeman 1967) and protein-calorie malnutrition imposed by restriction of total food intake (Chow, Lee 1964; Lee, Chow 1965; Seegers 1935; Srivastava et al. 1974) during pregnancy have been studied in the rat and shown to have a variety of effects on the young. As the protein level in the maternal diet was decreased from 25 to 10%, few effects were noted (Nelson, Evans 1953). Below 10%, effects were seen primarily in the dam. A study by Goldstein et al. (1979) using a diet containing 8 or 10% protein, for example, found little effect on the young. However, when the protein level was reduced to 5% or less, the diet affected the fetus as well as the pregnant dam. Under these circumstances, reproduction was characterized by a high incidence of resorption (Berg 1967; Curtiss 1953; Kosterlitz, Campbell 1957; Lee, Chow 1965; Nelson, Evans 1953; Seegers 1935; Zeman 1967) and neonatal death (Ambegaoker, Chandran 1959; McCoy 1940; Schultse, Halvorson 1949; Venkatachalam, Ramanathan 1966; Zeman 1967). Birth weight of the pups was also reduced. The reduction in birth weight was apparently primarily the result of a deficit in protein, even in animals restricted in total food intake, since the young of pair-fed animals did not differ from controls (Zeman 1967).

As shown in Table 1, size does not affect the various organs alike. When individual organs were weighed and compared to body size, it was found that the ratios of kidney weight and liver weight to body weight were reduced

Table 1. Relative Organ Weights at Birth[a,b]

	24% Casein	6% Casein
Crown to rump		
Length, mm	41.91 ± 2.15[c]	38.03 ± 2.76[d]
Relative organ weights[e]		
Liver, %	5.010 ± 0.618	4.530 ± 0.448[f]
Kidneys, %	1.014 ± 0.088	0.788 ± 0.135[d]
Heart, %	0.517 ± 0.073	0.595 ± 0.090[g]
Brain, %	3.877 ± 0.332	4.771 ± 0.656[f]
Thymus, %	0.163 ± 0.046	0.199 ± 0.632[g]

[a] From Zeman (1967).
[b] Eighteen rats/group.
[c] Mean ± one SD.
[d] $p < 0.001$.
[e] % of body weight
[f] $p < 0.05$.
[g] $p < 0.01$.

compared to those seen in controls, while the ratios of brain, heart, and thymus weights to body weight were increased. These data suggest that the brain, heart, and thymus were less affected while the kidney and liver were more affected by the prenatal protein deprivation (Zeman 1967).

Teleological reasoning could lead us to propose that these differences have some survival value for the young. The brain and heart are protected, whereas the kidney and liver -- organs considered to have a large reserve capacity -- appear to be reduced to a greater extent relative to body weight. However, the increased mortality rate of the young of protein-deficient dams (Zeman 1967) suggested the existence of effects other than simple reduction in organ size.

In the normal rat kidney at 18 d of gestation, the kidney has a few developing glomeruli and a few collecting tubules, but no proximal tubules are seen. At 19 d, proximal tubules can be identified, and at 20 d the proximal tubules as well as collecting tubules and developing glomeruli are more numerous. At term, there is little mesenchymal tissue remaining (Smith, Kissane 1963).

The morphology of the kidney of the newborn rat resembles the embryonic kidney in man and other species in which nephron formation is complete at term (Bogomolova 1966). The sequence of kidney development in the human (Potter 1965) includes the formation of S-shaped anlagen, flattening of the epithelial cells of Bowman's capsule, and later lobulation of the glomerulus.

Pigs, dogs, and rats that are fed protein-deficient diets postnatally show loss of cytoplasm in the proximal convoluted tubules, loops of Henle, and collecting tubules. Cloudy swelling is also seen in some cells of the proximal convoluted tubules, and their brush borders were only weakly positive in the periodic acid-Schiff (PAS) reaction compared to well-fed controls (Platt et al. 1964).

We postulated that prenatal protein-calorie malnutrition might affect the kidneys of the young in one of two ways: either causing pathological changes similar to those seen in malnourished animals, or, alternatively, causing a retardation in development.

MORPHOLOGICAL CHANGES

In the Neonate

Rats were mated and fed a diet containing either 24% or 6% casein throughout pregnancy. Longitudinal sections through the hilus of kidneys of newborn young of a control and a protein-deprived dam are shown in Fig. 1. The size difference in these sections is striking. In addition, kidneys from pups in the deficient group had a greater proportion of mesenchymal-like connective tissue and a reduced number of collecting tubules in the medullary rays.

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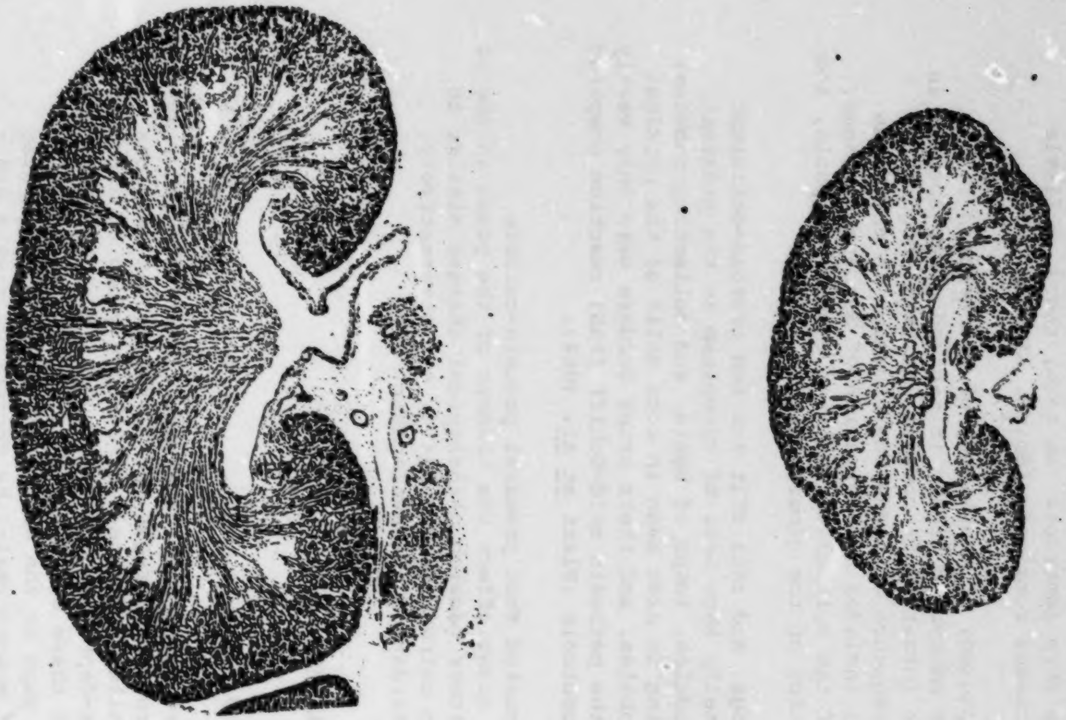

Fig. 1. Longitudinal sections through the hilus of kidneys of newborn young of dams fed 24% casein diet (left) and 6% casein diet (right). Hematoxylin and phloxine B. From Zeman (1968).

The glomeruli in one hilar section from each pup were counted. While not providing absolute numbers, the data indicated a reduction in the number of glomeruli compared to that seen in the controls (Zeman 1968). In addition, the relative proportion of immature glomeruli was increased. These glomeruli had more compact capillary tufts, and parietal epithelium remained cuboidal in many. S-shaped anlagen were more numerous.

There were fewer cross sections of proximal convoluted tubules per glomerulus in kidneys from the 6% casein group compared with the control group, suggesting fewer convolutions and also a shorter tubule. Tubular cells from the deficient group showed cloudy swelling, and the PAS staining of their brush borders was pale in comparison to the controls.

Since development of enzyme activity has also been used as an indication of maturity (Moog 1952; Verne, Hebert 1964), frozen sections of kidneys were stained for alkaline and acid phosphatase, adenosine triphosphatase (ATPase), nonspecific esterase, and leucine aminopeptidase. Acid phosphatase activity was found in the more mature proximal tubules in the control group but was not seen in the deficient group. Similar differences were seen in kidneys stained for alkaline phosphatase, but there were no differences between the two groups in esterase, ATPase, or leucine aminopeptidase activities.

The activities of some enzymes are known to decrease rather than increase during development (Hermann, Tootle 1964; Richter 1961; Stawe 1964; Wacker et al. 1961). However, activities of both acid and alkaline phosphatase have been shown to appear in larger areas of the developing rat kidney from birth to the age of 14 to 16 d, at which time they reach adult levels (Wachstein, Bradshaw 1965).

The effect of prenatal protein deprivation on cell division and cell growth in the young was also studied. Normal growth might theoretically consist of an increase in the number of cells, the size of cells, the amount of extracellular material, or a combination of these (Enesco, Leblond 1962; Winick, Noble 1955).

Kidneys of 18- and 20-d fetuses and newborn young of control and protein-deprived rats were investigated to obtain weight and content of DNA, RNA, and protein. Weights of kidneys from protein-deprived dams were significantly lower than controls. DNA was reduced at 20 d and thereafter, while RNA and protein contents were reduced beginning at 18 d. There were no significant differences between the two groups in ratios of weight to DNA, protein to DNA, or RNA to DNA (Zeman, Stanbrough 1969).

The DNA content of the diploid nuclei of rat cells has been reported to be a constant (Boivin et al. 1948; Davidson 1945; Mirsky, Ris 1949; Vendrely, Vendrely 1948). Therefore, a change in DNA content is thought to indicate a change in the number of cells, while the weight/DNA and protein/DNA ratios have been used to estimate cell size.

The similiarity of the curves for kidney weight and kidney DNA indicate that the decreased growth rate in the last 2 d of gestation resulted from a decreased rate of cell division. The slowing of the rate of cell division occurred at the same time in both groups, suggesting that the rate of cell multiplication was time dependent, not diet dependent.

The lower DNA content of the kidneys of the deficient group indicates a decrease in numbers of cells while cell size was not affected. The amount of RNA is considered to be associated with rapid protein synthesis (Caspersson 1950). The tendency for the amounts of RNA per cell and protein per cell to change in the same general direction in this study supports this concept. There was no difference between the two groups in RNA/DNA ratio in the fetal kidneys. The data suggest, therefore, that protein synthesis per cell occurred at the same rate in the kidneys of both groups.

To summarize, kidney sections obtained from the malnourished group did demonstrate some of the changes characteristic of postnatal protein-calorie malnutrition. At the same time, a major effect of intrauterine malnutrition appeared to be retardation in development. This was indicated by the increased amount of mesenchymal-like tissue, fewer glomeruli, a larger proportion of immature glomeruli, and fewer total cells. In addition, enzymogenesis, as indicated by decreases in acid and alkaline phosphatase activities, appeared to be retarded.

In Postnatal Young

The presence of retardation leads naturally to the question of the potential for "catch-up" growth and development in the postnatal period. To investigate that question, rats were mated and fed the 24% or 6% casein diet as described previously. When the animals littered, the litters were reformed. Three dams were mated at the same time. One was fed the control diet and one was fed the protein-deficient diet. The third rat, fed a stock diet, was larger and usually was producing its second litter. It was used as a foster mother during lactation to assure that an adequate milk supply was available to all pups. At birth, 5 pups from the control dam and 5 from the protein-deficient dam were given to the foster mother to suckle. All remaining pups not used for the study of newborns were discarded. All pups used were within the range of mean weight ± one standard deviation for the group. The litter of 10 constituted a normal-sized litter in these animals.

The effect of additional postnatal food intake was also investigated in animals in which the litter of 10 was cut to 4 (2 controls and 2 deficients) at 2 d of age. Pups were weaned at 21 d, caged individually, and fed a stock diet thereafter. The pups in the smaller litters increased in body weight compared to pups in litters of 10, demonstrating that reduction in litter size does result in increased food intake (Allen, Zeman 1971).

In studies of postnatal kidney morphology, the number of glomeruli per cross section and the number of cross sections of proximal convoluted tubules per glomerulus were obtained at birth, 13 d, and 22 d. Body weights, kidney weights, kidney DNA, and kidney protein were obtained at intervals from birth to 90 d of age.

As shown in Table 2, there were significantly fewer glomeruli (i.e., fewer nephrons) in kidneys from deficient animals at all ages studied (Allen, Zeman 1973b; Zeman 1967). The values given for newborns are less that those given earlier, since these are mature glomeruli only and these data were obtained from cross sections rather than longitudinal sections. Although some glomeruli were immature in newborn rats of both groups, with a larger proportion immature in the protein-deficient

Table 2. Postnatal Changes in Kidney Morphology[a]

	Control			Deficient		
Age	10/Litter	4/Litter	% Increase Due to Decreased Litter Size	10/Litter	4/Litter	% Increase Due to Decreased Litter Size
Glomeruli/hilar section[b]						
Newborn	44 ± 3.10	---	---	31 ± 3.60[c]	---	---
13 d	232 ± 8.10	228 ± 6.30	0.0	202 ± 4.60[c]	199 ± 4.70[d]	0.0
22 d	210 ± 5.80	203 ± 5.80	0.0	190 ± 2.50[c]	176 ± 3.70[d]	0.0
Proximal convoluted tubule cross sections/glomerulus[b]						
Newborn	4.94 ± 0.23	---	---	4.26 ± 0.22[c]	---	---
13 d	21.37 ± 1.02	24.29 ± 1.08	13.7	19.40 ± 0.83	23.15 ± 0.78[e]	19.3
22 d	34.29 ± 1.50	38.29 ± 1.78[f]	11.7	33.62 ± 1.32	39.66 ± 1.57[e]	18.0

[a] Adapted from Allen, Zeman (1973a).

[b] Mean ± SE; each value represents data from 10 animals.

[c] Significantly different from controls in litters of normal size, $P < 0.001$.

[d] Significantly different from controls in litters of equal size, $P < 0.001$.

[e] Significantly different from deficients in litters of normal size, $P < 0.001$.

[f] Significantly different from controls in litters of normal size, $P < 0.05$.

rats, no immature glomeruli were seen in the 13- and 22-d-old kidneys. Reduction in litter size had no effect on the number of glomeruli (Allen, Zeman 1973a). The apparent reduction in the number of glomeruli between 13 and 22 d is believed to be due to the increase in surrounding tubular tissue, causing the glomeruli to be more widely separated. It does not necessarily represent an actual decrease. The lack of nephrogenic tissue at Day 13 and the absence of an increase in the number of glomeruli in the smaller litters suggest that the deficit in number of glomeruli may be permanent.

Proximal tubule length, as indicated by the number of cross sections, was no longer decreased at 13 d in animals in either size litter. In both controls and deficients, the numbers of cross sections were greater in the smaller litters. The proximal convoluted tubules have also been shown to be the most responsive to the growth-stimulating effect of unilateral nephrectomy (Rollason 1949). Thus, it appears that the effect of prenatal protein deprivation on tubular size can be corrected postnatally.

The effects of postnatal diet on kidney weight gains from birth to 90 d of age are shown in Fig. 2. Kidney weight reached a plateau at about 60 d, except in the controls in small litters, in which it continued to increase. The difference in the plateau levels suggests a permanent difference in kidney size. During this plateau period, body weight continued to increase. Thus, the ratio of organ weight to body weight would be further depressed.

The total DNA, shown in Fig. 3, was reduced at all ages in pups of the protein-deficient group, a difference which endured despite the improved postnatal nutrition of the animals in the small litters. Protein content paralleled kidney weight and kidney DNA. Therefore, the protein-to-DNA ratio did not differ in the four groups.

The prenatal protein deprivation, then, caused a reduction in kidney cell number and nephron number that appears to have been permanent, since neither factor was increasing at 90 d of age. Cell size was unaffected by maternal diet or postnatal feeding.

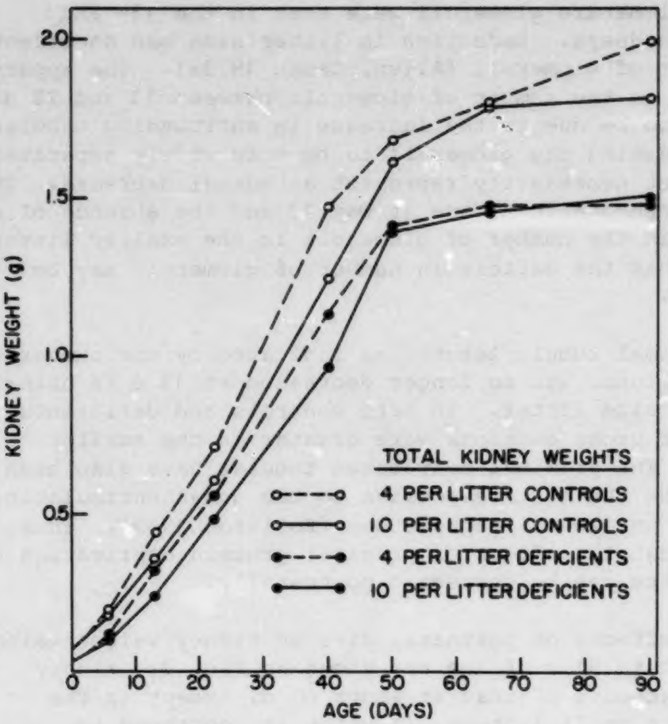

Fig. 2. Postnatal kidney weights. Each point represents data from 7 to 15 animals. Mean and SE values (g) for terminal points are: 4/litter: control = 1.979 ± 0.083, deficient = 1.456 ± 0.040; 10/litter: control = 1.775 ± 0.047, deficient = 1.511 ± 0.047. Data from control animals in normal-sized litters differ significantly from those in small-sized litters ($P > 0.05$). Each control group differs significantly from each deficient group ($P < 0.001$). From Allen, Zeman (1973a).

FUNCTIONAL ALTERATIONS

Kidney function and morphological and histochemical development have been found to be well correlated in normal postnatal development (Wachstein, Bradshaw 1965). The morphological changes described led us to expect changes in both glomerular and tubular function as a consequence of

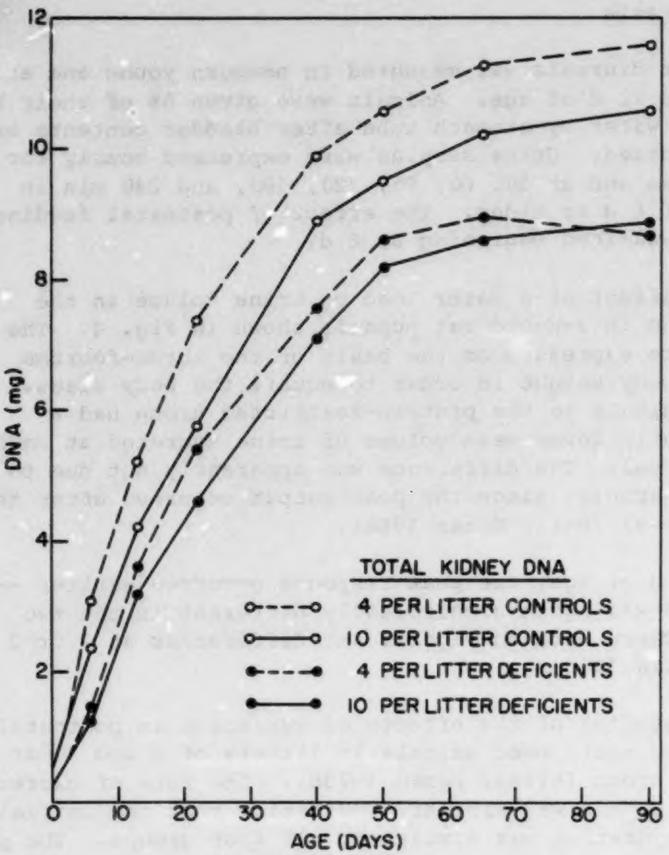

Fig. 3. Total kidney DNA content. Each point represents data from 10 animals. Mean and SE values (mg) for terminal points are: 4/litter: control = 11.495 ± 0.385, deficient = 8.820 ± 0.331; 10/litter: control = 10.605 ± 0.225, deficient = 8.982 ± 0.380. Data from control animals in normal-sized litters differ significantly from those in small-sized litters ($P < 0.005$). Each control group differs significantly from each deficient group ($P < 0.001$). From Allen, Zeman (1973a).

protein deprivation. Therefore, several aspects of function were investigated.

Water Diuresis

Water diuresis was measured in newborn young and at 2, 6, 13, and 22 d of age. Animals were given 5% of their body weight of water by stomach tube after bladder contents had been expressed. Urine samples were expressed hourly for 3 h in newborns and at 30, 60, 90, 120, 180, and 240 min in animals of 6 d or older. The effect of postnatal feeding was also examined beginning at 6 d.

The effect of a water load on urine volume in the newborn and in 2-d-old rat pups is shown in Fig. 4. The results are expressed on the basis of the three-fourths power of body weight in order to equate the body sizes. The newborn animals in the protein-restricted group had a significantly lower mean volume of urine excreted at each time interval. The difference was apparently not due to a delayed response, since the peak output occurred after the same interval (Hall, Zeman 1968).

At 2 d of age, the peak response occurred earlier -- at 1 h -- and was again significantly different in the two groups. There were no significant differences at 2 or 3 h (Hall, Zeman 1968).

The studies of the effects of variation in postnatal food supply again used animals in litters of 4 and 10 in each diet group (Allen, Zeman 1973b). The rate of excretion from 0 to 30 min was similar, indicating that the initial state of hydration was similar in all four groups. The peak rate of excretion occurred in 1 h in both groups. At 6 d, the deficient pups in both large and small litters had a marked reduction in peak urine flow. Reducing litter size did, however, increase the rate of urine production in the deficient pups. However, the total volume of urine excreted in 4 h was less in the deficients than in the controls (Fig. 5).

At 13 d and also at 22 d, the peak urine flow had increased further compared to that at 6 d (Fig. 5). There were no significant differences in excretion rates among the four groups (Allen, Zeman 1973b).

The change in units of measure should be noted. Data obtained from animals 6 d of age or older were expressed as a percentage of the total load excreted, rather than on the

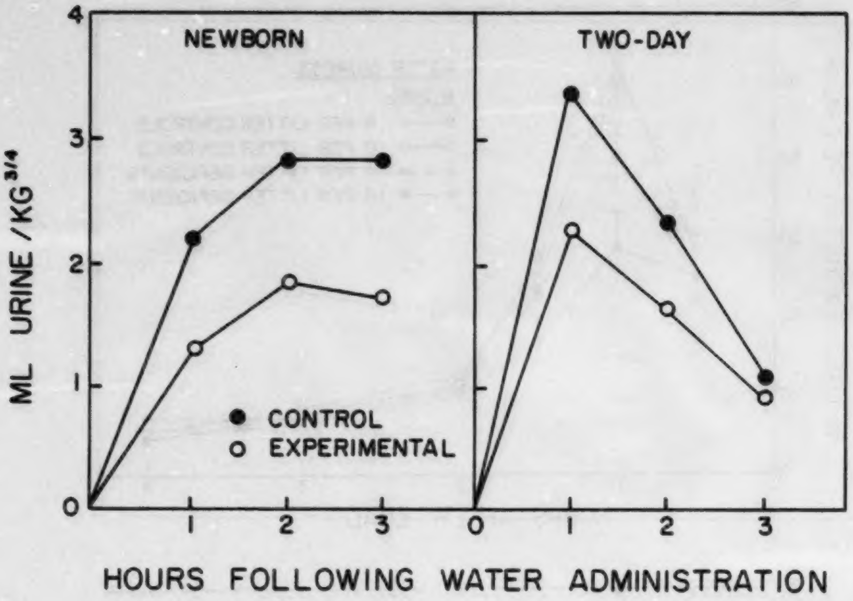

Fig. 4. Urine flow of newborn and 2-d-old rats during water diuresis.

basis of a power of body weight. Surface area (Smith 1951), total body water, and extracellular fluid space (Falk 1955) have also been suggested as standards of reference. Regardless of the basis for expression of results, it seems clear that 1) prenatal protein deficit results in a depression of the ability of the young to excrete a water load, and 2) the condition disappears with adequate postnatal feeding of survivors.

Response to Antidiuretic Hormone

The ability of the kidney to respond to antidiuretic hormone (ADH) was studied in large and small litters at 6 and 13 d. The bladder of each animal was emptied. Each animal was then given a subcutaneous injection of 10 mU ADH in normal saline per 100 g body weight followed by intragastric administration of deionized water equivalent to

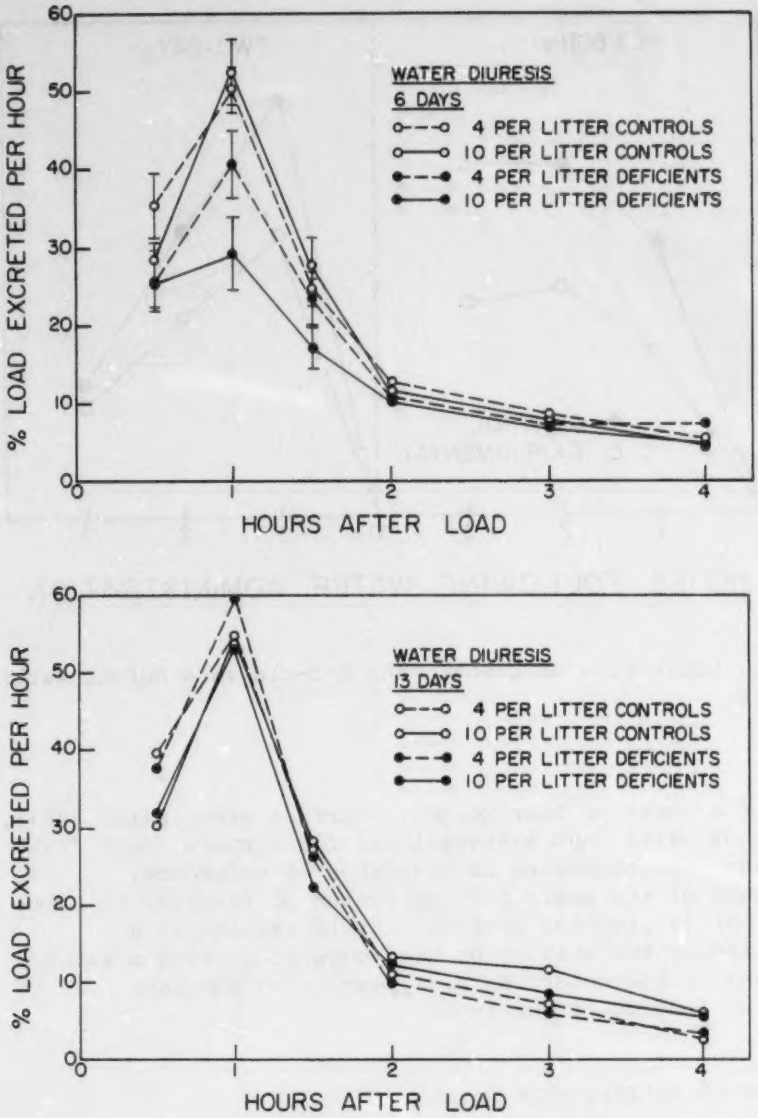

Fig. 5. Urine flow rates after water loading equivalent to 5% of body weight. Age of animals: Upper graph, 6 d; lower graph, 13 d. Each point represents data from 10 to 20 animals. Vertical bars show SEM. Adapted from Allen, Zeman (1973b).

5% of body weight. Urine was collected at intervals over a 3-h period.

In this experiment, it seemed most informative to compare the response to water loading in the presence and absence of ADH. At 6 d, flow was most depressed in the first hour in the controls in small litters; however, all rats showed some response (Fig. 6). At 13 d, the response of all groups was similar (Fig. 6). The response was greater and more rapid than that seen at 6 d (Allen, Zeman 1973b).

Falk (1955) and Adolph (1957) have shown that rats at 3 to 5 d of age are capable of some response to ADH; however, Heller (1952) observed none until 29 d. In the present study, the increased antidiuresis at 13 d indicates that the degree of response increases with age. It also suggests that 1) the depressed response in deficient pups at 6 d is a result of immaturity, and 2) this function in deficient pups matures to normal levels by 13 d.

Glomerular Filtration Rate

Glomerular filtration rate (GFR) was measured by determining inulin clearance in control and protein-deficient 6-d-old rats with a method adapated from that used by Falk (1955). A sufficient number of hourly blood samples could not be obtained from a single animal without hemodynamic side effects or death of the animal. Therefore, 4 littermates of approximately the same size were used to determine one clearance rate. Because of the variations in animal size, results were expressed per 100 g of body weight.

To obtain clearance rates in 22-d-old rats, we attempted a constant intravenous infusion. The method resulted in a wide range of values for GFR, possibly from wide variations in urine flow. Therefore, data were used only for those animals whose plasma inulin values could be plotted in straight line.

The values obtained were compared with inulin clearance at 22 d as determined by a single-injection technique. Data from that method were used only if peak plasma concentrations of inulin had been reached prior to the

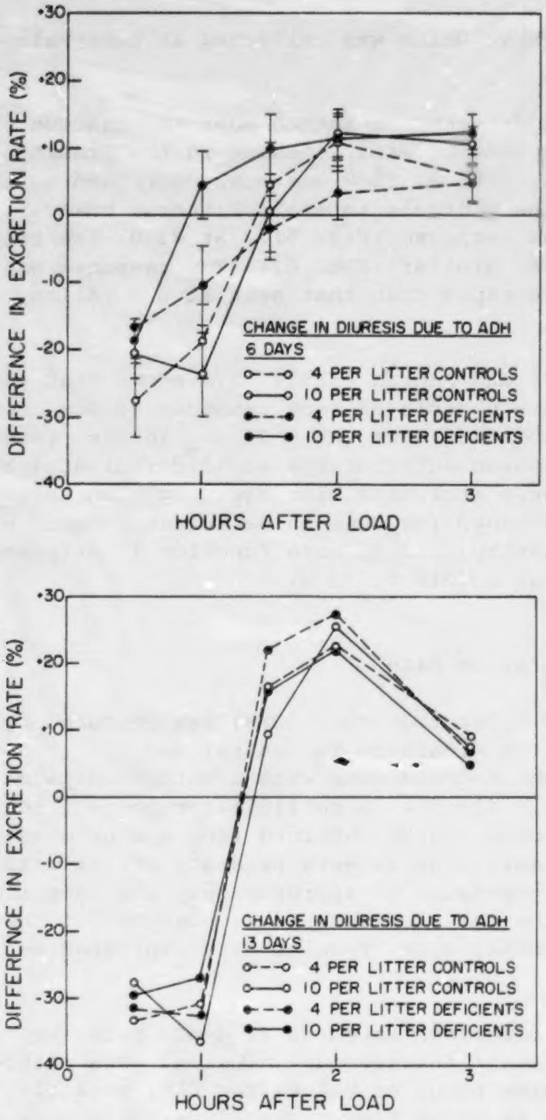

Fig. 6. Change in urine flow rate after ADH administration. Age of animals: upper graph, 6 d; lower graph, 13 d. Values for 6-d 10-per-litter deficients at 0.5 to 1.0 h after loading are significantly different from all other groups ($P < 0.001$). Adapted from Allen, Zeman (1973b).

beginning of the first clearance period, and if values fell in a straight line when plotted against time.

Regardless of the method used, GFR was markedly reduced in protein-deprived pups, a difference that persisted at least to 22 d (Table 3) (Allen, Zeman 1973b). When postnatal food intake was increased, GFR in deficient pups increased significantly compared to deficients in normal-sized litters but did not reach rates seen in control pups (Allen, Zeman 1973b). The similarity of values obtained by the two techniques in 22-d-old pups indicated that our results were valid.

Tubular Resorption

Tubular function was studied by several methods. In light of the importance of proximal tubular resorption in returning essential nutrients to the general circulation, we investigated amino acid transport in the renal tubules in control and prenatally protein-deprived animals at the end of gestation. A method adapted from Segal and Crawhill (1968) was used to study the *in vitro* accumulation of glycine and of the amino acid analog α-aminobutyric acid (AIB) in slices of renal cortex. Water content was determined by drying to constant weight; extracellular fluid space was determined by measuring the uptake of labeled inulin.

The intracellular fluid concentration of labeled amino acid was expressed as a distribution ratio, i.e., the ratio of intracellular to extracellular concentration. A distribution ratio of >1 indicates that there is active uptake of substrate against a concentration gradient by a mechanism other than diffusion.

There was no change over time in the total tissue water in kidney slices from either the control or protein-deprived group (Grosvenor, Zeman 1983). Extracellular water increased in control tissues over the 60-min incubation period. The increase in the protein-deficient tissue was not statistically significant. Neither total water content nor extracellular fluid content differed between the two groups (Grosvenor, Zeman 1983).

Table 3. Glomerular Filtration Rate in Control and Prenatally Protein-Deprived Rat Pups in Normal- and Reduced-Sized Litters[a]

		Control		Deficient	
Age	Technique	10/Litter	4/Litter	10/Litter	4/Litter
6 d	Single Inj.	0.271 ± 0.094 (12)	--	0.064 ± 0.019 (12)[b]	---
22 d	Single Inj.	0.442 ± 0.026 (12)	0.452 ± 0.014 (10)	0.275 ± 0.016 (11)[c]	0.336 ± 0.020 (10)[c,d]
	Const. Inf.	0.396 ± 0.031 (7)	0.422 ± 0.026 (7)	0.259 ± 0.021 (7)[e]	0.317 ± 0.021[e,f]

[a] Mean ± one SEM (ml/min/100 g). Number of subjects shown in parentheses.

[b] Significantly different from controls, $P < 0.001$.

[c] Significantly different from controls in normal-sized litters, $P < 0.001$.

[d] Significantly different from deficients in normal-sized litters, $P < 0.05$.

[e] Significantly different from controls in normal-sized litters, $P < 0.01$.

[f] significantly different from deficients in normal-sized litters, $P < 0.01$.

AIB was transported against a concentration gradient by samples from both groups (Table 4). The distribution ratio in the samples from deficient animals was greater than that of control samples throughout and, at 60 min, was almost twice that of controls. Glycine was also transported against a concentration gradient by samples from both groups, but the difference between the two groups was significant only during the first 15 min. At 60 min, uptake was the same in the two groups (Grosvenor, Zeman 1983).

Slices of kidney cortex contain primarily tubular tissue. Cortical slices have been used previously by several investigators to determine the proximal tubule transport mechanisms (Rosenberg et al. 1961). Recently, however, a technique for evaluating transport with isolated proximal tubules has been developed (Hellman et al. 1968), and the proponents of this model have criticized the use of cortical tissue slices for evaluating proximal tubule function. They have suggested that incubation of tissue slices does not allow adequate oxygenation to the interior cells. However, Roth et al. (1978) demonstrated that, when gassed with 95% oxygen in carbon dioxide, oxygen consumption in cortical slices was higher than in isolated tubules.

Others have questioned the use of slices on the grounds of lack of specificity of assessing luminal transport (Evers et al. 1976; McNamara et al. 1973). While basolateral transport may be involved, there is evidence that suggests that the uptake measured in cortical slices is primarily luminal (Evers et al. 1976; Lelievre-Pegorier, Geloso 1980; McNamara et al. 1973; Roth et al. 1978). First, the transport mechanism in the cortical slices is sodium dependent, as is the mechanism in the isolated tubules, while the basolateral mechanism is not sodium dependent (Evers et al. 1976; McNamara et al. 1973). Secondly, α-methylglucose, a nonmetabolizable glucose analog that is transported by these slices, has been shown not to interact with the basolateral membrane (Lelievre-Pegorier, Geloso 1980). Finally, the kinetic and developmental patterns of amino acid transport are the same in both cortical slices and isolated tubules (Reynolds et al. 1978).

Thus, the accumulation measured is considered to be the net effect of the influx of substrate into the cells and efflux of substrate out of the cells. Although the

Table 4. Distribution Ratio[a] of Amino Acid Accumulation by Renal Cortical Tissue Slices from Control and Prenatally Protein-Deprived Rat Fetuses[b]

	Incubation Period (min)			
	15	30	45	60
AIB				
Control	1.69 ± 0.1	2.52 ± 0.2	3.28 ± 0.8	3.86 ± 0.4[c]
Protein-Deficient	1.93 ± 0.1	3.09 ± 0.3	5.06 ± 0.7	7.07 ± 1.3[c,d]
Glycine				
Control	1.92 ± 0.2	3.53 ± 0.3	4.89 ± 0.4	6.54 ± 0.3[c]
Protein-Deficient	2.73 ± 0.3[d]	3.33 ± 0.6	5.88 ± 0.6	6.37 ± 0.7[c]

[a] DPM per ml intracellular fluid/DPM per ml extracellular fluid.

[b] Each value represents the mean of at least 6 determinations. From Grosvenor, Zeman (1983).

[c] Significantly increased from the value at 15 min, $P < 0.005$.

[d] Significantly different from control, $P < 0.025$.

mechanisms cannot be distinguished, this method does seem appropriate for assessing transport ability.

Glycine is transported across cell membranes by several different transport mechanisms, i.e., by the A transport system described by Christensen and co-workers (Christensen et al. 1973), by an imino acid mechanism, and by a system relatively specific for glycine (Hellman et al. 1968). The glycine accumulation observed in our study could be the result of transport by any or all of these mechanisms. Since glycine is a natural amino acid, part of the accumulation may also be due to metabolism or incorporation of glycine into the cells.

Although AIB is not a natural amino acid, it is apparently transported by the same mechanism as other amino acids. McInnes and Scriver (1979) demonstrated that AIB is involved in the mutual inhibition between amino acids and sugars and that its transport is sodium dependent, interactions that are characteristic of the natural amino acid transport system. Christensen and co-workers (Christensen et al. 1973) include AIB in their A transport system.

Differences in amino acid transport ability were based on a comparison of small kidneys from deficient animals with the significantly larger kidneys from controls. Although the cortical slices from deficient kidneys were smaller than those from controls, the size difference does not seem to have affected the results. Previously, Smith and Segal (1968) demonstrated that the distribution and uptake of amino acids were comparable in slices weighing 1 mg or more, and all of the tissue slices in the experiments described weighed at least 1 mg. Since fluid distribution was equivalent in the two groups, and since the accumulation of amino acids in both groups indicated uptake against the concentration gradient, differences in diffusion could not have caused the differences in accumulation.

The younger developmental age of the deficient kidneys may be the cause of their greater ability to accumulate AIB, since growing tissue such as regenerating liver (Christensen et al. 1948), neoplasms (Gale 1953), fetal intestine (Christensen, Streicher 1948), and fetal pancreas (Cheneval, Johnstone 1974) can accumulate higher concentrations of amino acids than their mature or normal counterparts. This same phenomenon may also occur in immature kidneys. Although amino aciduria is common in newborns, their renal tubule transport mechanisms are capable of accumulating greater concentrations of amino acids than are those of adults (Webber 1967). This is apparently due to an increased influx rather than a decreased efflux of amino acids. On a molecular level, this higher activity might be due to either an increase in the number of transport sites or an increase in the affinity of these sites for AIB.

Glycine accumulation in deficient kidneys does not appear to fit a model of developmental delay, since the accumulation of glycine in the deficient samples was equal to, not greater than, the accumulation in the control samples. However, the difference in the pattern of accumulation between AIB and glycine may be due to differences in the time of development of the transport mechanisms for AIB and glycine. Age-related differences in the development of amino acid transport mechanisms have been demonstrated by several investigators. For example, cystine and lysine present different patterns of development of their transport mechanisms in the kidneys of young rats (Segal, Smith 1969). The neutral and basic amino acids have also been shown to have different patterns of maturation. Baerlocher et al. (1971), using Long-Evans rats, demonstrated differences in the development of AIB and glycine transport mechanisms supporting the possibility that those mechanisms mature at different developmental ages.

The greater accumulation of glycine compared to AIB accumulation in control tissue may be due to the incorporation of glycine into the insoluble material of the cells, preventing glycine efflux. Glycine accumulation would then be greater than that of AIB, which cannot be incorporated and would move both in and out of the cells. On the other hand, the greater accumulation of glycine may indeed be due to differences in the maturation of the transport mechanisms. If the glycine transport mechanism develops late, it may be immature in both the control kidney and the developmentally delayed kidney slices from the deficient animal, explaining the equal accumulation of glycine in deficient and control kidney samples.

The greater accumulation of AIB by the deficient samples, possibly indicating greater tubular resorption of amino acids, could be important to protein-deprived pups, which have a high postnatal mortality. These pups are unable to suckle adequately (Zeman 1967), and their ability to digest and absorb protein is compromised (Zeman, Fratzke 1977). Thus, it seems reasonable to assume that conservation of available amino acids is important and may have survival value for the young.

Tubular Secretion

In a study of other tubular functions, maximal tubular secretory capacity was measured by determining the maximum transport capacity for para-aminohippuric acid (PAH). This tubular maximum for PAH (Tm_{PAH}) was measured at the same time as GFR by the single injection technique.

The plasma PAH concentration reached a peak at 40 to 50 min following injection and then began a surprisingly gradual decline; in some cases, the concentration seemed to be at a steady state. The data (Table 5) indicate that deficient progeny in normal-sized litters had significantly reduced Tm_{PAH} values compared to either control group, a difference that persisted when the results were expressed per 100 g body weight. Deficient progeny in small litters showed slightly increased maximum tubular secretory capacity, but the difference was not statistically significant when expressed on the basis of body weight.

No relationship was found between PAH secreted (mg/min/100 g) and plasma PAH concentration (Allen, Zeman 1973b). The tubular maximum for PAH appeared, instead, to be dependent on GFR. The values of Tm_{PAH} showed a significant linear correlation ($r = +0.79$; $P < 0.001$) between the two variables, implying that the ratio of Tm_{PAH} to GFR is constant at levels above threshold. The Tm_{PAH}/GFR ratio was slightly higher in control animals from normal-sized litters in comparison to controls from small litters, and significantly higher in deficient rats from small litters (Allen, Zeman 1973b).

The Tm_{PAH} values may have been reduced in these animals because of the deficit in nephron number or a retarded development of the proximal convoluted tubules. However, since Tm_{PAH} is dependent on GFR, it is possible that the tubular maximum was low simply because GFR was low.

Table 5. Tubular Maximum for Para-aminohippuric Acid in 22-Day-Old Rats[a]

	Control		Deficient	
	10/Litter	4/Litter	10/Litter	4/Litter
Tm_{PAH} (mg/min)	0.335 ± 0.018 (11)	0.445 ± 0.031 (10)	0.163 ± 0.014 (10)[b]	0.233 ± 0.018 (10)[b,c]
Tm_{PAH} (mg/min/100 g)	0.653 ± 0.031 (11)	0.703 ± 0.045 (10)	0.419 ± 0.035 (10)[b]	0.489 ± 0.042 (10)[d]
Tm_{PAH}/GFR	1.535 ± 0.070 (11)	1.396 ± 0.099 (10)	1.651 ± 0.079 (10)	1.384 ± 0.082 (10)[e]

[a] Mean ± one SEM. Number of subjects shown in parentheses.

[b] Significantly different from controls in normal-sized litters, $P < 0.001$.

[c] Significantly different from deficients in normal-sized litters, $P < 0.01$.

[d] Significantly different from controls in normal-sized litters, $P < 0.01$.

[e] Significantly different from deficients in normal-sized litters, $P < 0.05$.

The tubular maximum for glucose has also been described as being dependent on GFR and unrelated to plasma glucose levels. Van Liew and co-workers (1967) suggest that previous investigators who did not observe this phenomenon used animals with considerably less variation in GFR than that found in rats. In addition, Tm_{PAH} has conventionally been expressed per milliliter of glomerular filtrate. This practice would obscure the effects of variation in GFR.

It is possible that the true tubular maximum for PAH secretion was not reached in either the control or protein-deficient groups. In addition to its dependence on GFR, which is much lower in 22-d young than in the adult, other problems are presented by the fact that GFR has been shown by Heller (1951) to vary with the state of hydration in both young and protein-deficient rats.

While our Tm_{PAH} data can be accepted only with some reservations, they are probably valid as a basis for comparison among the groups, since all dosages were based on body weight and all collections were made at the same time.

Increased postnatal food intake resulted in an increased Tm_{PAH} (Allen, Zeman 1973b). This increase cannot be attributed to an increase in the number of nephrons, since there was no increase in nephron number when litter size was reduced. Instead, it is possible that the increased postnatal feeding led to more rapid maturation of the proximal convoluted tubules. Tubule length is increased, probably by an increase in the numbers of cells, as mentioned previously. As a consequence, the amount of secretory tissue was probably increased. At the same time, Tm_{PAH} may have increased consequent to the increased GFR. The lower Tm_{PAH}/GFR ratio in the animals in the smaller litters suggests that increased postnatal food intake affected Tm_{PAH} less than it did GFR.

Although function tests were not performed on animals older than 22 d, it is possible that prenatal protein deficit has a permanent effect on renal function. The permanent reduction in nephron number might be expected to influence function. In addition, extrarenal factors affecting function, such as hormones and plasma protein, may also be affected by prenatal protein deficiency.

SUMMARY

Our studies show that prenatal protein deprivation has a long-term (if not permanent) effect on kidney morphology in the young and effects on renal function that persist at least to the end of weaning. Since animals are still alive and growing, we might ask whether this has any real physiological significance. At this point, it is important to mention that 50% of the young born of protein-deprived dams in these studies did not survive more than 3 d. Those animals that were the subject of the study of postnatal kidneys were presumably less affected. We do not know the extent to which compromised renal function contributed to neonatal death in the others.

These results can be extrapolated to other species only with great caution, since the rat kidney is relatively immature at birth. It is probable that increased postnatal feeding has a more beneficial effect on these rapidly developing tissues than it would have in species in which the kidneys are more mature at birth. Yet, kidney function did not reach the control level in spite of improved postnatal nutrition. Compromised renal function in the more mature kidneys of other species may be less amenable to postnatal recovery and -- at the very least -- leave the kidneys less able to respond to postnatal stress factors.

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DETECTION OF RENAL DYSFUNCTION IN NEONATAL RATS: METHODOLOGIES AND APPLICATIONS

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INTRODUCTION

The kidneys of adults are the primary target organ for many toxicants. This occurs because many of the same physiological processes that enable the kidneys to assume their important roles in maintaining homeostasis also expose the renal parenchyma to relatively high levels of potentially harmful chemicals. Included in these processes are a high rate of blood flow, carrier-mediated transport mechanisms, P-450-dependent drug metabolizing enzymes, the large intraluminal surface areas of the tubules (particularly the brush-border-lined proximal tubule), and the increased concentration of chemicals that occurs as water is removed from the glomerular filtrate during passage through the medullary gradient. Therefore, much attention has been paid to the evaluation of renal function in adult toxicology studies, and the methodologies and relative test sensitivities have been reviewed by several authors (Balazs _et al_. 1963; Berndt 1976; Kluwe 1981).

Little attention, however, has been paid to such assessments in neonatal animals that have received either direct or transplacental exposure to suspected renal toxicants. Zeman and co-workers (Zeman 1968; Hall, Zeman 1968; Allen, Zeman 1973), working with maternal protein restriction, were among the first to publish accounts of physiological alterations in renal development subsequent to _in utero_ insult. More recent studies have examined the effect of maternal administration of methyl salicylate on the development of the renal papilla (Woo, Hoar 1972); the effect of *in utero* exposure to dinoseb, paraquat, and TCDD on the uptake of organic anions and phlorizin binding capacity of renal tissue slices (Gibson 1976); the morphological and histochemical retardation in fetal kidney development as a result of maternal administration of the herbicide (2,4,5-trichlorophenoxy)acetic acid (Highman *et al*. 1977); the morphological and functional sequelae of prenatal exposure to dinoseb, a herbicide known to at least delay renal development in the fetus (McCormack *et al*. 1980); the effect of streptozotocin-induced gestational diabetes on the development of renal tubular functions in the progeny (Rolig *et al*. 1982); and the developmental profile of *in vitro* ion transport and the ability of young adults to respond to water deprivation and volume expansion following prenatal exposure to methyl mercury (Smith *et al*. 1983).

METHODOLOGIES

As rodents are generally the species of choice in toxicology studies, researchers in developmental nephrotoxicity have been restricted in their methodological approaches by the relatively small body sizes of their target population. The work that has focused on the *in vitro* approach for evaluating nephron function lacks true comparability to the *in vivo* situation and evaluates only a small fraction of nephron functions. The other approach generally utilized -- waiting until the pups have grown to sufficient size to permit a more classical type of functional evaluation to be performed -- allows a long interval to elapse before assessment of toxic implications, and thus may not be well suited for detecting subtle alterations in development. Several years ago, we became interested in physiological approaches for evaluating renal dysfunction in neonatal rodents, and have devised several simple yet sensitive measures of overall functional competence. The kidney provides an exceptionally well suited system for such study because its function is well understood in terms of morphology, and because the urine, which represents the end product of its physiological function, can be easily collected and analyzed using noninvasive techniques.

The basis of our approach lies in the ability to quantitatively collect urine from neonates over discrete time intervals and to measure important urinary constituents. Occasionally we obtain serum samples from the experimental subjects and perform routine biochemistry on them, as well. The advantage of obtaining both serum and urine samples is that clearance and fractional excretion values can be calculated using standard equations. However, given the small blood volumes of the pups, serum collection is an invasive technique that can be done only at the conclusion of an experiment. Micturition is induced in the pups by a technique similar to that described by Falk (1955), which takes advantage of the fact that, for the first two weeks of life, rat pups cannot micturate voluntarily, but require some external stimulus to void their bladders. The pups are held ventral side upward and gently stroked in the perianal region with a microcapillary tube. This action results in a reflex constriction of the bladder and expulsion of urine. Urine expressed in this fashion can be quantitatively collected in the capillary tube. (For pups of between 2 and 4 wk of age, small metabolism cages constructed of 50-mm funnels and plastic beakers can extend the range of this technique out to weaning, when the use of regular metabolism cages becomes feasible.) After capping the tubes with sealant and briefly centrifuging them to remove air bubbles, the volume of urine is determined by measuring the length of the column of fluid. Thus, by isolating the pups from the dams, the exact amount of urine produced over a given time period can be determined by first voiding the bladder, placing the pups in 250-ml Griffen beakers that contain bedding to a depth of about 5 cm, and voiding the bladders and collecting the urine at timed intervals. Because rat pups are poikilothermic during early postnatal development (Adolph 1957), the beakers are placed in a water bath to a depth of 1 to 2 cm and the temperature of the surface of the bedding is maintained at 32 to 34°C.

Urinary constituents that we routinely record include osmotic pressure (by vapor pressure osmometry), pH, chloride (by coulometric titration), sodium and potassium (by ion-specific electrodes), glucose (by the oxygen rate method), creatinine (by the Jaffe rate method), urea (by the enzymatic conductivity rate method), and protein (by the Bio-Rad procedure). In serum, we measure osmotic pressure, chloride, sodium, potassium, glucose, creatinine, and urea.

All procedures are adapted for small sample sizes, either directly or through dilutions or standard addition procedures. Thus, we are able to obtain virtually complete profiles on volumes as small as 60 µl.

Table 1 lists the function tests that we currently utilize. Because the kidney is known to possess a large functional reserve capacity, many of the tests apply some form of stress to the system, such as fluid deprivation or hormonal challenges, in order to determine maximal responses. By doing this, we hope to be able to detect small deficits in functional ability that are not apparent under basal conditions. Briefly, the water diuresis test involves a per os administration of distilled water (5% v/w) and measuring the volume of urine produced over the next 40 to 80 min. The desmopressin acetate (DDAVP, an analogue of arginine vasopressin that lacks pressor activity) response test is applied in two fashions. In the first, water-loaded pups receive a subcutaneous dose of 100 mU/kg DDAVP and the

Table 1. Renal Function Tests Applicable to Neonatal Rats

Noninvasive	Invasive
Water Diuresis	*In Vitro* Transport
Desmopressin Acetate Response	Clearance Values Creatinine Free Water Electrolytes
Hydropenia Response	
Diuretic Respose	T(M) Glucose
Electrolyte Excretion	Serum Chemistries
pH (Titratable Acids)	Kidney Weight
Proteinuria	Histology Nephrogenesis Pathology

inhibition of urine production is recorded. In a second procedure, the same dose of DDAVP is given to induce maximal concentrating ability in fluid-deprived pups and the osmotic pressure of the urine is recorded. The hydropenia test is similar to this latter DDAVP test, but here the endogenous concentrating ability is tested by recording the urine volume and osmotic pressure during the period of 4 to 6 h after pups have been removed from the nest and hence deprived of fluid intake. In the diuretic response tests, the function of discrete segments of the nephron is tested by administering pharmacological agents such as acetazolamide, furosemide, chlorothiazide, and amiloride and monitoring the quality and quantity of urine over four consecutive 90-min periods. In the glucose tolerance test, pups receive a large challenge dose of glucose and the amount of sugar appearing in the urine between 15 and 30 min after injection is determined. Invasive tests generally include the obtaining of serum samples, and -- when combined with information obtained from the urine specimens -- allow us to calculate a variety of clearance values. Tissues are routinely examined for pathology, and degree of nephrogenesis (remembering that this process occurs largely postnatally in the rat) is determined by counting the number of glomeruli in mid-hilar cross sections. Finally, we perform *in vitro* studies on the ability of cortical slices to accumulate prototype organic anions (p-aminohippurate), cations (tetraethylammonium bromide), and sugars (α-methylglucopyranoside). Details of these procedures can be found in previous publications (Kavlock 1982; Kavlock, Gray JA 1982; Kavlock, Gray JA 1983; Daston, Kavlock 1982; Kavlock *et al*. 1983; Gray, Kavlock 1983; Daston *et al*. 1983).

APPLICATIONS

There are two basic problems that must be addressed in the field of abnormal functional development: 1) Are alterations in functional development more sensitive indicators of developmental toxicity than alterations in morphological development? 2) If so, what are the sensitivities of various function tests? What is needed to address these issues is a model system that is free of morphological lesions but that has demonstrable functional alterations. Largely because of the newness of this field, such models simply do not exist. Yet, without them, the second problem is unapproachable. Similarly, without sensitive procedures, it is impossible to determine whether functional alterations exist. To get around this paradox, we decided to administer adult renal toxicants directly to newborn pups and apply our tests at time periods when renal dysfunction would be expected. While the treatment period was different from our desired prenatal time period, at least it would tell us something about the usefulness of the various test procedures that we were developing. It soon became apparent, however, that newborn rats are quite resistant to several classic renal toxicants. Based upon acute toxicity data and on pathology and functional measurements, newborn pups tolerate much higher levels of both mercuric chloride and sodium fluoride than do adult rats (Kavlock 1982; Daston et al. 1982; Daston et al. 1983). Thus, this approach did not demonstrate the utility of our test procedures. Our attention then turned to determining whether we could detect functional alterations in kidney development for compounds that are renal teratogens: Would our tests show toxic effects at dose levels below those producing overt lesions? Thus, we began working with the alkylating agent chlorambucil (Monie 1961) and the herbicide nitrofen (Costlow, Manson 1982). We were able to demonstrate that doses of chlorambucil sufficient to induce terata did alter renal function in neonates (Kavlock 1982; Kavlock, Gray JA 1983), but lower doses apparently did not. Prenatal administration of nitrofen seemed to provide us with many of the characteristics of a positive control (i.e., it induces alterations in renal functional development in individuals that do not appear to be affected on the gross morphological level), and data derived from it will be used to illustrate our approach.

Nitrofen (2,4-dichlorophenyl-p-nitrophenyl ether) is a preemergent herbicide that induces somewhat unusual forms of developmental toxicity. Ambrose et al. (1971) were the first to observe that dietary exposure during gestation to levels of nitrofen that were without effect on the dam decreased pup survival after birth. Kimbrough et al. (1974) confirmed this effect on pup survival and presented evidence that some unexplained perturbation in lung development prevented their normal expansion in the immediate postnatal period. Later, Stone and Manson (1981) and Costlow and Manson (1981) examined the nature of the perturbation in greater detail and concluded that a combination of cardiac malformations and diaphragmatic hernias induced by exposure to nitrofen during gestation prevented the successful transition to extrauterine life. They also noted a high incidence of hydronephrosis in surviving offspring. Finally, it has been reported that gestational exposure retards the morphological and biochemical differentiation of the brain, lungs, liver, and kidneys (Kavlock et al. 1982).

In the studies that will be discussed here, pregnant rats of several strains were given nitrofen by oral gavage in a corn oil solution during various periods of organogenesis. The original work was done in the Sprague-Dawley rat, and the treatment period encompassed the period of major organogenesis; subsequent studies used other strains and involved shorter treatment periods centered around the time when differentiation of the metanephric blastema was occurring. In all cases, the dams were allowed to give birth and rear their natural litters. The ages at which pups were examined are designated by PD (for Postnatal Day). Periodically the litters were examined for viability and growth, and dead or moribund pups, when observed, were removed from the cage and necropsied. Renal function tests were applied at various ages; at the conclusion of the tests, the pups were killed and their kidneys were weighed, fixed, and examined for pathology. Hydronephrotic pups were handled separately in the data analysis, and we wish to emphasize those effects that were found in the morphologically normal offspring. Often the same test was applied to pups of different ages in an effort to determine the persistence of functional alterations. These data were then subjected to two-way analysis of variance procedures (age and dose are the main effects in the models). Invariably the effects proved consistent across ages (i.e., there were no significant age X dose interactions). Therefore, for simplicity of interpretation, we will present the least-squares means pooled across ages.

RESULTS AND DISCUSSION

In the first experiment (Table 2), doses of 0, 4.17, 12.5, and 25 mg/kg/d were administered to Sprague-Dawley rats on Days 8 to 16 of gestation (Kavlock, Gray 1983). Offspring viability during the first two days of extrauterine life was severely reduced at the two higher doses (69 and 37% versus 99% at the control and low dose). Dying pups were generally found to have diaphragmatic

Table 2. Renal Function in Offspring of Sprague-Dawley Rats Exposed to Nitrofen on Gestation Days 8 to 16[a]

Observation	Dose (mg/kg/d) 0	4.17	12.5	25
Body Weight (g)	11.8	11.7	10.5*	8.5*
Kidney Weight (mg)	84	76	72*	62*
Glomerular Counts[b]	162	157	153*	123*
Water Diuresis[c] (%/40 min)	31	29	21*	15*
DDAVP Response (%/40 min)	13	12	13	6*
Hydropenia Response[d]				
Basal Osmolality (mOsM)	317	293	383	616*
Deprived Osmolality (mOsM)	726	666*	657*	712

[a] Data are pooled least-squares means from PD 3 and 6. Means that are significantly different from control value ($P < 0.05$) are denoted by an asterisk (*).

[b] The number of glomeruli present in a typical mid-hilar cross section.

[c] Response to 5% (v/w) per os water load on PD 3. In addition to the water load, DDAVP pups received a subcutaneous injection of DDAVP (100 mU/kg).

[d] Ability to excrete an osmotically concentrated urine following 6 h of fluid deprivation on PD 6.

hernias as the only identifiable lesion. Upon pathological examination, hydronephrosis was detected in 1/40 and 1/20 pups in the 12.5 and 25 mg/kg/d groups, respectively. Pups in these two high dose groups were growth-retarded, had reduced numbers of glomeruli, and were deficient in their ability to excrete a water load and to respond to injections of antidiuretic hormone when tested on PD 3. More interesting results, however, were observed in the hydropenia tests applied on PD 6. Here, pups in the 4.17 mg/kg/d group achieved only 92% of the urinary osmotic concentration found in control pups that were fluid deprived for 6 h, despite no evidence of gross pathology or growth retardation. This lack of ability of nitrofen-exposed pups to conserve water during hydropenia was our first demonstration of the ability to detect prenatally induced renal dysfunction in apparently morphologically normal offspring.

In the second experiment (Table 3), Long-Evans rats received a single dose of 75 mg/kg on Day 11 of gestation (Kavlock, Gray LE 1983a), a treatment regimen shown by Costlow and Manson (1981) to induce hydronephrosis. This treatment did not affect litter size at birth or postnatal growth and viability. Hydronephrosis was present in 23% of the treated offspring that were examined, as compared with only 1.5% of control offspring. Hydronephrotic pups performed quite poorly in tests of renal concentrating ability on PD 3 and 6. Treated pups that were not hydronephrotic also had a diminished ability to concentrate urine in the fluid deprivation tests, and showed significant increase in urine volume and solute excretion compared to controls. These deficits in organ function occurred despite any apparent retardation of histological differentiation of the kidneys on PD 8. At PD 50, treated and control animals were subjected to a 24-h fluid deprivation test. Deficiencies in renal concentrating ability were again noted, but this time only in animals that were later found to be hydronephrotic. Thus, nitrofen was again shown to be a renal functional teratogen in neonatal animals that are otherwise apparently morphologically normal; however, it appears that this effect may have been transient.

In the third experiment (Table 4), Sprague-Dawley rats received a single dose of 50 or 100 mg/kg on Day 11 of gestation. The high dose resulted in a 36% reduction in perinatal viability and a transient growth retardation (present through PD 6). Offspring in the low-dose group were comparable to controls in these respects. Hydronephrosis was observed in 2% of the control pups, 19% of the 50 mg/kg group, and 15% of the 100 mg/kg group. This lesion was first noted as a dilation of the renal pelvis in neonates and progressed with age to extensive involvement of

Table 3. Postnatal Renal Functional Effects in Long-Evans Rats Following Exposure to Nitrofen on Gestation Day 11[a]

Observation	Dose (mg/kg) 0	75
Body Weight (g)	10.0	9.9
Kidney Weight (mg)	166	171
Hydropenia Response		
Basal Osmolality (mOsM)	310	348
Final Osmolality (mOsM)	689	652*
Urine Volume (µl/6 h)	117	155*
Solute Excretion (µOsM/6 h)	66	81*

[a] Data are pooled least-square means from PD 3 and 6. Hydropenia response test is described in Footnote d, Table 2. Means that are significantly different from control value ($P < 0.05$) are denoted by an asterisk (*).

the renal cortex. Renal function was evaluated using a hydropenia response test on PD 2, 15, and 29, a DDAVP response test on PD 6 and 14, and a test of *in vitro* renal cortical slice transport ability on PD 3, 17, and 31. As observed in the Long-Evans rat, the hydronephrotic Sprague-Dawley pups were deficient in renal concentrating ability. However, they showed normal *in vitro* cortical transport functions. Nonhydronephrotic offspring were also deficient in renal concentrating ability. In this test, the time course of elaboration of a concentrated urine was followed for four 90-min periods following removal from the nest on PD 6 and 14. At the end of 180 min, the pups received a subcutaneous dose of 100 mU/kg DDAVP. Pups in both treatment groups began to show a deficit in

Table 4. Renal Concentrating Ability of Neonatal Sprague-Dawley Rats Exposed to Nitrofen on Day 11 of Gestation[a]

	Dose (mg/kg)		
Observation	0	50	100
Body Weight (g)	20.2	20.2	18.8
Urine Osmolality (mOsM)			
0 min	364	404	438*
90 min	551	489	546
180 min	841	753*	758*
270 min	943	881*	880*
360 min	1006	931*	887*

[a] Data are pooled least-square means from PD 6 and 14. In this concentrating test, urinary osmotic pressure is measured every 90 min following removal from the nest. At 180 min, pups receive a subcutaneous dose of 100 mU/kg DDAVP to test maximal elicitable response. Means that are significantly different from control value ($P < 0.05$) are denoted by an asterisk (*).

concentrating ability at the 180-min period, and this deficit was not corrected by administration of exogenous antidiuretic hormone. Thus, prenatal exposure to nitrofen was again effective in altering the renal functional capacity of apparently morphologically normal individuals. Since a deficit in renal concentrating ability could be due to a central (altered vasopressin secretion) or renal (inability to generate or utilize a medullary gradient) effect, the persistence of the deficit after administration of DDAVP indicates that the lesion induced by prenatal administration of nitrofen resides within the kidney itself, rather than at some extra-renal site.

In the last experiment (Tables 5 and 6), Fischer 344 rats were exposed to doses of 0, 10, 20, 40, or 80 mg/kg/d on Gestation Days 10 to 13 (Kavlock, Gray LE 1983b). All offspring in the two highest dose groups died within 24 h of birth. In dead or moribund pups from the high-dose group that were recovered from the nest, 50% had diaphragmatic hernias and 33% showed delayed development of the renal papilla. At the next lowest dose, the incidences were 37% and 7%, respectively. Very few pups died in the control and two low-dose groups, and a diaphragmatic hernia in 1 of 2 pups in the 20 mg/kg/d group was the only malformation observed in those groups during the neonatal period. However, when offspring were examined at weaning, 4 of 29 had diaphragmatic hernias, 1 female had a missing uterine horn, and 1 male had undescended testes in the 20 mg/kg/d group. In the 10 mg/kg/d group, 1 of 35 offspring had a diaphragmatic hernia. Animals in both treatment groups had

Table 5. Renal Concentrating Ability of Neonatal Fisher 344 Rats Exposed to Nitrofen on Gestation Days 10 to 13[a]

	Dose (mg/kg/d)		
Observation	0	10	20
Body Weight (g)	9.6	9.3	8.5*
Kidney Weight (mg)	105	101	91*
Hydropenia Response			
Basal Osmolality (mOsM)	294	295	251*
Final Osmolality (mOsM)	682	646*	650*
Urine Volume (µl/6 h)	100	107	124*

[a] Data are pooled least-square means from PD 3 and 9. Means that are significantly different ($P < 0.05$) from control value are denoted by an asterisk (*). Hydropenia response test is described in Footnote d, Table 2.

Table 6. Renal Function in Weanling Fischer 344 Rats Exposed to Nitrofen on Gestation Days 10 to 13[a]

Status, Observation	Dose (mg/kg/d) 0	 10	 20
Hydrated			
Body Weight (g)	105	101	86*
Fluid Intake (ml/100 g/d)	14.8	15.3	15.0
Urine Volume (ml/100 g/d)	6.8	6.7	5.6*
Urine Sodium (mmol/100 g/d)	1.3	1.2	1.2
Urine Potassium (mmol/100 g/d)	2.6	2.6	2.9*
Urine pH	6.5	6.8	7.1
Dehydrated			
Body Weight (g)	98	93	80*
Kidney Weight (mg/100 g)	102	103	107*
Urine Volume (ml/100 g/d)	3.4	3.4	3.0*
Urine Sodium (mmol/100 g/d)	0.7	0.6	0.9
Urine Potassium (mmol/100 g/d)	1.5	1.5	1.8*
Urine pH	6.8	7.2	7.6
Serum Urea (mg/dl)	12.8	12.4	15.1*

[a] Animals were placed in metabolism cages on PD 32 to 35. Data are analyzed by two-way (age X dose) analysis of variance. Data from hydrated animals are averaged over two 24-h periods. Dehydrated state represents 24 h of fluid deprivation. Means that are significantly different from control value ($P < 0.05$) are denoted by an asterisk (*).

smaller Harderian glands. Growth was suppressed in pups in the 20 mg/kg/d group at all observation periods. There was no evidence that this exposure regimen induced hydronephrosis in any surviving offspring. Neonatal kidney weights were reduced at this dose, but the effect was related to overall growth retardation. By weaning, absolute kidney weights were still reduced, while relative kidney weights were significantly greater than the control value.

This increase in relative organ weight could not be attributed to any pathological condition of the kidneys. Removal of the body weight effect by covariate analysis indicated that the kidneys were indeed enlarged in treated animals, but the effect was no longer significant. Thus, prenatal exposure to nitrofen under this exposure regimen induces a persistent overall growth retardation, but it is difficult to determine whether there is any specific effect on renal growth.

The renal concentrating ability of the offspring was tested on PD 3 and 9. Pups in the 20 mg/kg/d group had lowered basal urinary osmotic pressures, increased urine flows during hyropenia, and decreased maximal urinary osmotic pressures. Decreased maximal urinary osmotic pressures were also observed in pups in the 10 mg/kg/d group and, as noted before under other treatment regimens, this effect was present in the absence of any overall growth retardation or morphologically detectable renal lesions.

At 32 to 35 d of age, offspring from each dose group were placed in Nalgene metabolism cages; for two 24-h periods these animals were allowed free access to water, and urine production and urinary sodium, potassium, and pH were recorded. During the third 24-h period, water bottles were removed from the cages and the quantity and quality of urine produced under hydropenic conditions were recorded. At the conclusion of this period, the animals were killed and serum was collected for electrolyte and urea determinations. Regardless of the state of hydration, animals in the 20 mg/kg/d group excreted relatively smaller volumes of more alkalotic urine and greater amounts of potassium than did control offspring, but there were no differences in plasma electrolytes. Serum urea concentrations were elevated in the high-dose group, although the increase was relatively small (25%). No similar effects were noted in animals in the low-dose group. Thus, we now have been able to establish that alterations in renal function that are induced prenatally can persist beyond the immediate neonatal period, although the relationship between the renal impairment and the growth retardation, if any, is by no means clear. Body growth could be suffering from less than optimal renal function, or renal growth could be compromised by some other factor that also affects overall growth.

CONCLUSIONS

Several tests of renal function that are applicable to neonatal rats have been presented. These tests do not rely on sophisticated equipment; they are rather simple to apply, and in many instances the subjects can be reused in other tests, making the tests economical, as well. Using the herbicide nitrofen as a model compound, we have demonstrated that these function tests are more sensitive to alterations in development than are morphological examinations. Exposure to nitrofen during *in utero* development resulted in renal dysfunction in three strains of rats using treatment periods as short as a single day of gestation. While the results between strains cannot be directly compared to each other because of the various lengths of treatment, it appears that nitrofen may have a high chronicity factor in producing its effect: As the treatment period became longer, the dose required to induce either morphological or functional changes became smaller. Of the many tests available to assess renal function, the hydropenia test, which evaluates an integrated and demanding aspect of overall functional competence, seems well suited as an initial examination of renal function in young animals. Recording additional parameters (beyond osmotic pressure and urine volumes) in this test may give clues as to the site or mechanism of a functional deficit. The inability of DDAVP to abolish the renal concentrating defect in the Sprague-Dawley rat points to a non central, non collecting duct site of effect, while the lack of effect on transport mechanisms observed in cortical slices points away from the proximal tubule. Following this logic, the effect may reside in the loop of Henle or distal tubule. The elevation in urinary pH noted in weanling Fischer 344 rats exposed to nitrofen on Gestation Days 10 to 13 may be related to a respiratory alkalosis resulting from an effect on pulmonary function (see Raub *et al*. 1983). The elevated potassium excretion could be related to this alkalosis, or may indicate an effect on electrolyte homeostatic mechanisms, perhaps at the level of aldosterone (which might again point to an effect on the distal tubule). The relatively lower urine volumes in treated animals may be related to an overall reduction in GFR, as serum urea levels were significantly elevated in those animals.

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NEONATAL METHYL MERCURY EXPOSURE: BIOCHEMICAL MECHANISMS IN THE DEVELOPING KIDNEY

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INTRODUCTION

Perinatal methyl mercury poisoning is characterized by neurological disturbances including tremors, dysarthria, ataxia, loss of vision and hearing, insomnia, and general mental retardation (Hunter et al. 1940; Takeuchi 1972). Because of these neurological disturbances, pathological actions of the organomercurial on development of the central and peripheral nervous systems have represented a major focus of methyl mercury research, and it is clear that methyl mercury causes a variety of morphological, histological, and biochemical changes in central and peripheral neural tissues (Shiraki, Takeuchi 1971; Reuhl, Chang 1979). However, methyl mercury also reaches high concentrations in organs such as kidney, which may then also be a target for perinatal methyl mercury toxicity. In this regard, it has been reported that exposure to methyl mercury during development results in alterations of kidney ultrastructure and function secondary to tissue injury (Gyrd-Hansen, Helleberg, 1976; Chang, Sprecher 1976). The current paper identifies some of the biochemical mechanisms underlying effects of methyl mercury in the developing kidney.

THE ORNITHINE DECARBOXYLASE/POLYAMINE SYSTEM

One biochemical tool that has proven useful in determining the mechanisms by which exposure to environmental contaminants causes developmental alterations

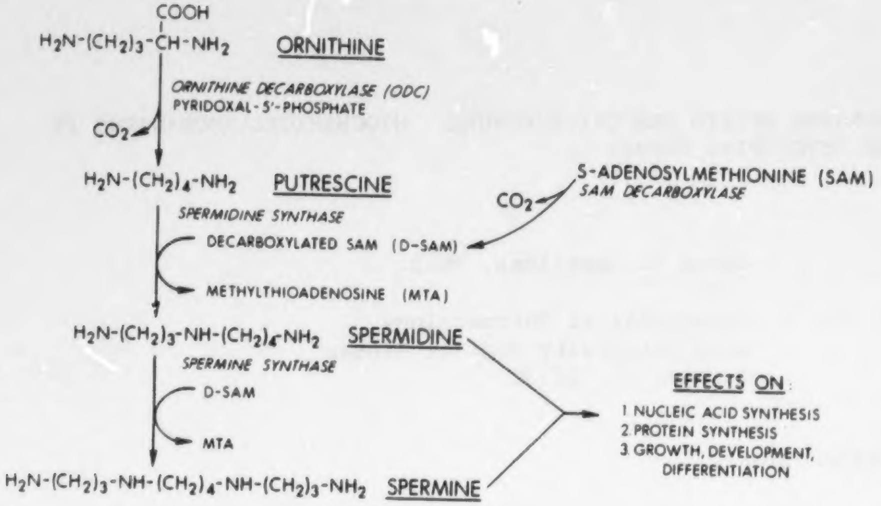
COOH
$H_2N-(CH_2)_3-CH-NH_2$
ORNITHINE
ORNITHINE DECARBOXYLASE (ODC)
PYRIDOXAL-5'-PHOSPHATE
CO_2
$H_2N-(CH_2)_4-NH_2$
PUTRESCINE
S-ADENOSYLMETHIONINE (SAM)
SAM DECARBOXYLASE
CO_2
SPERMIDINE SYNTHASE
DECARBOXYLATED SAM (D-SAM)
METHYLTHIOADENOSINE (MTA)
$H_2N-(CH_2)_3-NH-(CH_2)_4-NH_2$
SPERMIDINE
SPERMINE SYNTHASE
D-SAM
MTA
EFFECTS ON:
1 NUCLEIC ACID SYNTHESIS
2 PROTEIN SYNTHESIS
3 GROWTH, DEVELOPMENT, DIFFERENTIATION
$H_2N-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$
SPERMINE

Fig. 1. Ornithine decarboxylase/polyamine system.

has been the ornithine decarboxylase (ODC)/polyamine system (Fig. 1). ODC and its polyamine end products spermidine and spermine are major regulators of RNA and protein synthesis during tissue growth, and each organ has specific development patterns of ODC and polyamines (Slotkin 1979; Abraham, Pihl 1981). Numerous studies have demonstrated the utility of this system in rapid and sensitive detection of perturbations of cellular growth and development by exposure to drugs, hormones, or environmental toxins in the perinatal period (Slotkin 1979; Russell 1980).

Before assessing how methyl mercury acts on the kidney ODC/polyamine system, it is necessary to examine the general role of ODC and the polyamines in kidney growth during normal development.

An important new tool in establishing direct participation of the ODC/polyamine system in development is the recently available drug α-difluoromethylornithine (DFMO), a potent, selective, and irreversible inhibitor of ODC (Metcalf et al. 1978). DFMO has been shown to decrease the growth of a variety of tumor and cell lines, to inhibit liver regeneration after partial hepatectomy, and to arrest early embryogenesis of mammalian species. In the current studies, DFMO administration in vivo has been used to explore the potential roles of ODC and the polyamines in development of the kidney in the neonatal rat.

Administration of a single dose of DFMO (500 mg/kg s.c.) to 2-d-old rats produced a prompt and profound decrease in kidney ODC activity, and inhibition was still nearly total at 24 h post injection. Consequently, a daily dosage schedule of 500 mg/kg was adopted for studies of chronic effects of DFMO on kidney development; this treatment caused a prompt, effective, and persistent inhibition of ODC activity. Daily administration of DFMO to neonatal rats had no detectable effect on body weight gains over the first 2 wk postpartum. Thereafter, although the DFMO-treated animals continued to gain weight, they did so at a much reduced rate over the ensuing 2 wk; most notable was the attenuation of the growth spurt normally occurring during the 4th wk after birth. Despite normal body weights through 14 d of age, rats given DFMO had kidney weights that were significantly subnormal by 7 d of age and became progressively more subnormal with continued treatment (38% below control values by 28 d of age). These results support the view that the ODC/polyamine system participates directly in the growth and development of the neonatal mammalian kidney.

The question may arise as to whether DFMO exerts its growth-inhibitory effects through actions on the ODC/polyamine system or through direct cytotoxic actions. Since DFMO is an amino acid, it could conceivably act directly through protein synthesis inhibition. However, direct measurement of [^{14}C]leucine incorporation into tissue proteins of developing rats indicated no such direct effect of DFMO even at 2-4 h post injection, when its actions would be maximal. Additionally, studies in adults indicate absence of direct cytotoxic or antimitotic activity by DFMO, as no systemic toxicity can be detected even after prolonged treatment. It is highly likely, then, that the effects of DFMO on kidney growth reflect its actions on ODC/polyamine-mediated processes.

If the early tissue growth deficits caused by DFMO are directly related to effects on polyamine levels, then it is apparent that putrescine and/or spermidine are major components involved in these deficits. Measurement of tissue polyamine levels indicated that DFMO reduced the concentration of putrescine, the first product of the pathway, almost immediately; spermidine was affected within a few days of initiating treatment, but the spermine concentration was virtually unaffected or actually increased. These results in neonatal rats resemble those seen in tissues from mature animals, in which spermine is relatively refractory to acute change (Danzin et al. 1979; Bartolome et al. 1982b). Indeed, the maintenance of spermine levels may explain why some tissue and body growth still occurs despite marked deficiencies of putrescine and spermidine. In any case, these studies indicate that the ODC/polyamine system plays a major role in modulating growth processes in the developing mammalian kidney.

It is apparent, then, that perturbations of ODC activity and/or polyamine levels during early development (when active DNA synthesis and mitosis are taking place) are especially deleterious to kidney growth and function in the developing rat. Consequently, we next addressed whether the effects of perinatal methyl mercury on kidney maturation could be related to primary effects of the organomercurial on the developmental pattern of ODC.

EFFECTS OF METHYL MERCURY ON ORNITHINE DECARBOXYLASE DEVELOPMENTAL PATTERN

Neonatal rats were given methyl mercury hydroxide in doses spanning the range from subtoxic to frankly toxic exposure (1, 2.5, or 5 mg/kg s.c. daily, beginning the day after birth and continuing for up to 20 d). Previous studies conducted in our laboratory showed that these doses cause profound neurochemical alterations regardless of whether body weight gains are affected (Bartolome et al. 1982a; Bartolome et al. 1983). Methyl-mercury-exposed rats showed an early, pronounced, and persistent increase in ODC activity, and the effect was reversed shortly after methyl mercury administration was discontinued at 21 d of age. Since ODC levels are highest during peak periods of cellular proliferation and differentiation, the ODC elevations produced by methyl mercury treatment represent a prolongation of the time in which these processes occur. Accordingly, one would predict that perinatal methyl mercury would produce renal overgrowth. This was, in fact, observed; kidney weights became elevated by 7 d of age, and values were still 25% above control at 40 d. The effects were prominent even in the absence of generalized toxicity, since body weight gains were not affected by doses of methyl mercury which altered renal ODC and growth. Examination of the nucleic acid content and histological characteristics indicated that the renal overgrowth was a classical hypertrophy, with elevations of RNA but not DNA, and no evidence of hydronephrosis.

POSSIBLE BIOCHEMICAL MECHANISMS

How do methyl-mercury-induced changes in the ODC system come about? The pattern of ODC activity in the normal kidney typically shows a surge after 3 wk of age, corresponding to the onset of action of neuronal and hormonal trophic factors that influence kidney development (Grignolo et al. 1982). Since kidney ODC levels of methyl-mercury-treated animals were markedly elevated early during development, we considered it important to examine the possibility that these enhanced ODC levels and the persistent renal hypertrophy are caused by changes in the ontogeny of tissue responsiveness to endogenous neuronal or humoral trophic agents. Consequently, we examined first the potential role of sympathetic nerves in altered tissue growth, utilizing chemical sympathectomy with 6-hydroxydopamine (6-OHDA). Neonatal administration of 6-OHDA produced a rapid, profound (>90%), and long-lasting destruction of peripheral sympathetic nerves. This sympathectomy completely inhibited the initial phase of hypertrophy observed in the preweaning stage, indicating that sympathetic nerves do, in fact, participate in initiation of methyl-mercury-induced kidney hypertrophy. However, 6-OHDA did not influence the later, more pronounced phase of renal hypertrophy, indicating major participation of other effects of methyl mercury unrelated to neuronal actions. We then addressed whether alterations in hormonal responses were responsible for the later phase of hypertrophy, using acute stimulation of ODC activity as an index of renal response to these trophic stimuli. In the normal rat, kidney ODC responses to growth hormone, angiotensin, vasopressin, isoproterenol, and serotonin were absent at birth and matured only after 3 to 4 wk of postnatal life. However, neonatal rats treated with methyl mercury showed marked ODC responses to these same agents as early as 10-19 d of postnatal age. These results are consistent with the hypothesis that a temporal shift in the kidney's sensitivity to endogenous trophic stimuli occurs in methyl mercury toxicity and may contribute to renal hypertrophy and subsequent abnormal function.

In order to find out whether the enhanced ODC sensitivity of the kidney observed in methyl-mercury-treated animals constitutes a general biochemical action of the organomercurial, similar studies were conducted in the liver. This tissue was chosen because, in contrast to the kidney, liver ODC responses to the same trophic agents are present early in development (Grignolo *et al*. 1982). Unlike the results obtained for the kidney, methyl mercury treatment failed to cause generalized enhancement of liver ODC responses, and in fact suppressed some of the responses. Again, these results are in keeping with subsequent effects on tissue growth, since methyl-mercury-treated rats display deficits in liver weights, not hypertrophy.

CONCLUSIONS

Neonatal methyl mercury exposure markedly alters the biochemical maturation of the kidney, as shown by shifts in the ontogenetic pattern of ODC, a sensitive and reliable index of cellular development. Because of its role in polyamine biosynthesis and hence in cellular growth, multiplication, and differentiation, ODC may ultimately provide a link between specific actions of methyl mercury on cellular metabolism and abnormalities of tissue development and function. Methyl mercury may produce its effects on the developing kidney secondarily through alterations in trophic stimuli contributing to kidney maturation. The compound should not be considered a specific neurotoxin; rather, it displays a spectrum of effects on developing organs.

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DISCUSSION SUMMARIES--KIDNEYS

NORMAL ORGANOGENESIS OF THE HUMAN KIDNEY
(Wallace W. McCrory)

DR. SLOTKIN: Is there evidence that you're actually inducing transport sites with the penicillin, or is there a change in affinity, or perhaps a change in the efficiency of transport (possibly by a change in ΔpH across the tubule)?

DR. SPITZER: It's a change in the number of receptors. That's based mainly on work by Hook. A finite number of receptors can be formed, and you're speeding up the process. Once the total number has been reached, nothing happens further.

DR. SLOTKIN: Is there any evidence as to what is the signal that communicates to the cell that it needs to make more sites?

DR. SPITZER: It's very attractive to consider diet as an inducer of this maturation process. We've looked for one with bicarbonate, thinking that the shift from mother's milk to regular food would induce the bicarbonate transport system. That's not always the case. It appears that this is somehow printed in the cells, and develops at that point regardless of change in diet.

CHEMICALLY INDUCED POLYCYSTIC KIDNEY DISEASE
(John F.S. Crocker *et al*.)

DR. CAROLE KIMMEL: When did you administer the DEHP to produce the cystic kidneys?

DR. CROCKER: We took an eluate of plastic dialysis tubing in solution and fed it 3 times weekly at equivalent doses. We started with 4-wk-old animals and continued treatment for a year.

DR. WILLIAMS: DEHP is teratogenic in mice and rats. Are there any effects of DEHP during kidney development?

DR. CROCKER: Short-term experiments in the pregnant animal have not been done.

DR. SLOTKIN: Are there differences in the timing of the appearance in the glucocorticoid receptors in the different strains?

DR. CROCKER: We have not looked.

DR. McCRORY: The original rabbit model had a polycystic disease that was correctable or preventable by potassium administration. Is this the case in your model?

DR. CROCKER: Giving potassium chloride coincident with the glucocorticoid dramatically decreases cyst induction. Giving potassium as a bicarbonate or other salt does not have the same effect.

DR. BALLARD: Two of the properties that you've presented are not consistent with the receptor-mediated phenomenon. One is the dose. I think the binding is through another protein besides the receptor. Secondly, a receptor specificity for different steroids should reflect the binding specificity; in your list, they don't seem to correspond. For these reasons, I think it's overly speculative to invoke the endogenous corticosteroids in the etiology of this disease.

DR. CROCKER: But I think we'll be able, as we inbreed further, to dramatically decrease the dose.

DR. SMITH: It's surprising that a couple of steroid acetates work and nonacetates don't. Could it in fact be an acetate effect, and by linking a steroid onto it you make it more accessible to the cell?

DR. CROCKER: Different acetate salts given alone do not reproduce these effects.

THE CONTRIBUTION OF THE KIDNEY TO THE RETENTION OF SODIUM DURING GROWTH (Adrian Spitzer)

DR. LEVINE: By way of follow-up on blood pressure, there are at least two reports (a 4-yr follow-up and a 7-yr follow-up) indicating an inverse relationship between birth weight and blood pressure.

DR. SPITZER: There is evidence suggesting that one can identify the population at risk of developing hypertension very early in life. Kaas and co-workers have shown that babies of hypertensive mothers tend to have higher (although not necessarily abnormal) levels of blood pressure. Studies done in England and Canada indicate a tendency toward inheritance of hypertension.

DR. McCRORY: Jose Strauss showed that puppies are less responsive to angiotensin. So caution is needed in interpreting the high PRA levels of the newborn. Also, in rats, if the sympathetic system is blocked with spontaneous hypertension, blood pressure remains within normal limits. So whether the high renin is a primary or secondary phenomenon needs to be established.

DR. SPITZER: If the newborn's vasculature responded to angiotensin as does the adult's, all newborns would be hypertensive. But they're not, so the peripheral vessels must be less sensitive to this substance. The relevant questions, in the context of sodium homeostasis, are whether the adrenal gland responds to angiotensin and the renal tubule responds to aldosterone to the same extent as in the adult.

DR. SLOTKIN: The Japanese group that first developed this rat strain did a whole series of studies on the effects of various antihypertensive drugs on these animals. As I recall, they found that the diuretic agents were much less effective in preventing hypertension than were sympatholytics or agents that destroy sympathetic neurons. Doesn't that argue for a neurogenic rather than a hormonal mechanism of hypertension?

DR. SPITZER: I don't think there's just a single mechanism. There is no question that hypertension cannot occur if the kidney responds appropriately to changes in blood pressure. Guyton has provided conclusive evidence of a direct relationship between the level of blood pressure and sodium excretion. In the normal individual, this helps to maintain the blood pressure within the normal range by regulating the intravascular space. Therefore, one always invokes some defect in the reabsorption of sodium and water in a hypertensive patient.

DETECTION OF RENAL DYSFUNCTION IN NEONATAL RATS: METHODOLOGIES AND APPLICATIONS (Robert J. Kavlock and George P. Daston)

DR. SPITZER: What is the cause of hydronephrosis in these animals?

DR. KAVLOCK: I don't know. It happens quite early in development, and we have wondered whether it might be some inductive effect. We have not checked for an obstruction.

DR. McCRORY: I think the easiest way to do that is by an IVP. I use a dental X-ray machine to do IVP's on young rats and mice. One can also measure resistance to flow through the ureter. Secondly, are these kidneys small for the animals' body sizes? Do you know anything about the animals' eating habits? Is their ability to concentrate less because they eat less?

DR. KAVLOCK: The kidney weights are not different when corrected for body weight. On a relative basis, treated animals eat the same amount of food after weaning.

DR. SPITZER: Did you do any tests of proximal tubule function?

DR. KAVLOCK: In Dr. Daston's experiment, we did cortical slice uptakes, which yield at least some information about the proximal tubules, and they were normal.

DR. SPITZER: As you said before, a deficit in concentrating ability can arise from a variety of factors. This makes it difficult to identify the site of the lesion.

DR. KAVLOCK: The hydropenia response is not a mechanistic test, but it can give a clue that something is wrong. It's not an end in itself.

DR. McCRORY: In human infants, one of our first observations was that their concentrating ability appeared to be impaired. Subsequently, this turned out to involve a series of problems: the maturity of the collecting ducts, ADH responsiveness, GFR -- all all these factors are crucial. The most crucial, however, is the amount of urea present to be concentrated within the interstitium, and it's very well known that low-protein diets blunt ADH response and that, conversely, high-protein diets increase ADH response. So that's not a valid index of morphologic abnormality or of functional change.

DR. KAVLOCK: We assume that, during the preweaning period, the mothers provide them with nutritionally identical diets.

DR. McCRORY: To interpret the results on potassium, one needs to know the animals' intake of water. It's difficult to conclude "excessive mineral loss" without knowing the mineral intake.

DR. KAVLOCK: The animals were on the same diet. They ate the same amounts of food (compensated for body weight).

DR. McCRORY: Were the urea excretion rates, adjusted for weight, comparable in the nonresponding and responding animals?

DR. KAVLOCK: We examined that in the adults, and the rates were similar.

NEONATAL METHYL MERCURY EXPOSURE: BIOCHEMICAL MECHANISMS IN THE DEVELOPING KIDNEY (Jorge V. Bartolome)

DR. SPITZER: We tend to think of methyl mercury as a poison to the kidney, but obviously that's not the case, at least at this level. What are the toxic levels, as compared to the doses you used?

DR. BARTOLOME: In the adult rat, the LD_{50} is approximately 15 mg/kg; in the neonate, the LD_{50} is pretty close to that: 15-20 mg/kg. The highest dose we used was 5 mg/kg. Altered developmental patterns were observed even in animals treated with 1 mg/kg.

DR. GRAY: You saw hindlimb paralysis in animals treated with 5 mg/kg, so you're not far from the toxic dose.

DR. BARTOLOME: Hindlimb paralysis only appeared after 2-3 wk of age in animals given 5 mg/kg. As I said before, animals exposed to 1 or 2.5 mg/kg did not show overt signs of neurotoxicity.

DR. SPITZER: What are the effects of these doses of mercury on subcellular organelles?

DR. BARTOLOME: It has been reported in the literature that administration of doses of methyl mercury similar to the ones used in our study produces marked morphological alterations of the proximal tubules in both adult and developing animals.

DR. SPITZER: The fact that the kidney's bigger doesn't mean necessarily that it's normal.

DR. BARTOLOME: That's true.

DR. CAROLE KIMMEL: This fits the pattern that Dr. Slotkin described: precocious development eventually leading to deficits in organ function at later ages. A lot of the effects reported in the literature are delayed effects: you may not see all the changes in behavior until later during development, and they can become more severe as the animals age.

DR. McCRORY: I didn't hear anything about function. I agree with Dr. Spitzer that you've got a bigger kidney, but

I don't know what it means. Can't you measure PAH or some other indicator of proximal tubule function?

DR. BARTOLOME: We are planning such studies.

DR. DASTON: Have you measured mercury levels in any of these kidneys at any ages? We've done quite a bit of work with inorganic mercury toxicity during early postnatal development and have found striking changes in the toxicity of mercuric chloride over the period of renal maturation during the first 3 wk of life. There is almost no toxicity in the neonate, and then toxicity increases during development. In fact, the neonate requires about 6 times as much mercury to produce the same effects as the 3-wk-old. Also, there's a decrease in the amount of mercury present in the kidney.

DR. BARTOLOME: We have not yet done such studies. Determinations of tissue levels of mercury have been repeatedly done by several investigators; the tissue that mostly concentrates methyl mercury is the kidney, followed by the brain and the liver. One of the next questions we will address is whether a correlation exists between concentrations of mercury and the effects on the biochemical maturation of the kidney already discussed.